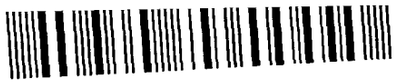
Characterisation of *Rhizoctonia solani* anastomosis groups and their pathogenicity to potato

Thesis



CHARACTERISATION OF *RHIZOCTONIA SOLANI* ANASTOMOSIS GROUPS AND THEIR PATHOGENICITY TO POTATO

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Abstract

The significance of different anastomosis groups (AGs) of *Rhizoctonia solani* in potatoes was investigated. One hundred and thirty-five isolates of *R. solani* were collected from potato crops in Britain and assigned to an AG. The predominant group was AG3PT but isolates of AG2-1 and AG5 were also present. Identification of AG was aided by the development of a PCR assay specific for AG3PT. AG5 was also isolated from a couch grass plant present in a potato sample. Hyphal fusion between isolates of AG2-1 and different lengths of the rDNA IGS1 region indicated diversity amongst the AG2-1 isolates. Pathogenicity tests, glasshouse and field experiments were also undertaken comparing potato disease caused by different AGs. AG3PT was associated with black scurf, root infection, severe stem and stolon infection including stem pruning. Severe stem infection was also observed with AG5, but stem pruning, black scurf and root infection were uncommon. Two types of infection were observed amongst isolates of AG2-1. One set of isolates, characterised by a shorter version of the IGS1 regions, caused frequent small lesions no longer than 5 mm, on potato stems. Isolates of AG2-1 with a longer version of the IGS1 region could cause severe stem canker and pruning. Severe root infection was observed in plants infected with AG8 but little other sign of disease. In field experiments yield losses were greatest in plants infected with AG3PT and AG8, suggesting that root infection is a key factor in determining yield losses. Isolates collected in this study, in addition to other isolates, were characterised for thiamine requirement, hyphal growth rates under different temperatures, sensitivity to different fungicides, and *in vitro* production of sclerotia. The rDNA ITS region of several isolates were sequenced and analysed, confirming the presence or absence of subgroups in AGs associated with potato.

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Statement of advanced studies

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Publications

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Contents

List of Tables

List of Figures

1. Introduction

1.1 The fungus *Rhizoctonia solani*

Rhizoctonia solani (Kühn) is one of many important phytopathogens belonging to the in the genus *Rhizoctonia.* This genus consists of many ubiquitous soil-borne fungi that cause damage on a multitude of plant species (Menzies, 1970). *Rhizoctonia* species are also well known for their mycorrhizal association with orchids, amongst other plant species (Andersen and Rasmussen, 1996). Additionally, some *Rhizoctonia* isolates are antagonistic against noted phytopathogens (Sneh, 1996) and some are important in nutrient cycling as saprophytes. Within the *Rhizoctonia solani* species complex at least 13 anastomosis groups (AGs) and numerous subgroups exist (Carling *et al.,* 2002a). Each AG has a very different life history and is believed to be genetically distinct (Cubeta and Vilgalys, 1997).

1.1.1 Classification

Rhizoctonia species are grouped into the genus *Rhizoctonia* on the basis of vegetative characteristics. However, the sexual state of many *Rhizoctonia* species, including *R. solani*, is known. The teleomorph of *R. solani* is *Thanatephorus cucumeris* (Frank) Donk. Analyses of the teleomorphs within the genus *Rhizoctonia* indicate that the genus contains members from different families, orders and even classes (Ogoshi, 1996).

The *Rhizoctonia* genus is characterised by the following attributes (adapted from Ogoshi, 1987; Sneh *et al.,* 1991):

1. Branching near the distal septum in young, vegetative hyphae
2. Hyphal constriction and septa formation located near the origin of branches
3. Dolipore septum
4. Lack of clamp connections, rhizomorphs or conidia
5. Undifferentiated sclerotia

The name *Rhizoctonia solani* is now conserved (Stalpers *et al.,* 1998; Roberts, 2000) and is used throughout this study. *Thanatephorus* is in the family Ceratobasidiaceae, Order Ceratobasidiales in the Class Basidiomycota (Roberts, 1999). Within the classification of Roberts (1999), the Ceratobasidiaceae consist of three genera, *Ceratobasidium*, *Waitea* and *Thanatephorus*. *Ceratobasidium* is distinguished from the other genera by differences in hymenial and basidial shape. *Thanatephorus* species are distinguished from *Waitea* species in that they possess indeterminate sterigmata often longer than 6 μm in length and basidiospores capable of self-replication. Species of the *Waitea* genus possess determinate sterigmata up to 6 μm in length and self-replicating basidiospores (Roberts, 1999). It is often observed that isolates of *Ceratobasidium* are mono- or bi-nucleate whereas the other two genera are bi- or multi-nucleate, consequently some members of *Ceratobasidium* are commonly referred to as binucleate *Rhizoctonia*. Hyphal maturity and genetic state can influence the nuclear content of cells, therefore basidia can be considered the critical attribute in the classification of the *Ceratobasidiaceae.*

1.1.2 The life cycle of *Rhizoctonia solani*

Basidia are the key characteristic of basidiomycete fungi and are integral to the generation of genetic variation. In a typical basidiomycete, a diploid nucleus is formed within the basidia, which then undergoes meiosis to form four haploid nuclei. Each haploid nucleus is then incorporated into a basidiospore that when released from the basidia, germinates to form primary mycelia. These primary mycelia are monokaryotic and haploid. Two sets of these primary mycelia fuse (anastomosis) forming a secondary mycelia (the heterokaryon). This heterokaryon typically contains two genetically distinct haploid nuclei per hyphal compartment. This heterokaryon is the dominant phase of the life cycle in most basidiomycetes (Moore and Novak Frazer, 2002). Field isolates of *R. solani* have been found to be heterokaryotic (Flentje *et al.,* 1970).

Maintaining the heterokaryotic state requires that the mitotic division of each haploid nuclei is synchronised so that each type is present in progeny cells. To aid this, typical basidiomycetes form a hyphal structure known as a clamp connection to manage nuclear sorting. *Rhizoctonia* species lack clamp connections and are nearly always multinucleate whereas typically, basidiomycete heterokayons are binucleate (dikaryotic). These attributes coupled with poor *in vitro* sporulation mean that analysis of sexual (mating) compatibility in *Rhizoctonia* is more demanding compared to other basidiomycetes as the identification of heterokaryotic mycelia is difficult.

The presence of tufts has been used as an indication of the presence of heterokaryons for some AGs. These tufts are rapidly growing aerial heterokayotic mycelia where the colonies or primary mycelia merge (Bolkan and Butler, 1974; Whitney and Parmeter, 1963), and have been identified in AG1 (Whitney and Parmeter, 1963), AG4 (Anderson *et al.,* 1972) and AG8 (Yang *et al.,* 1992). Amplified Fragment Length Polymorphism (AFLP) analysis has supported observations of the heterokaryotic state within these tufts (Julian *et al.,* 1999). Identifying the heterokaryon by the presence of tufts is not ideal as tuft-like growth can occur between heterokaryons (Yang *et al.,* 1993) and do not always occur where heterokaryosis is present (Cubeta *et al.,* 1993; McCabe *et al.,* 1999).

Despite these disadvantages, Anderson *et al.* (1972) used tuft presence in conjunction with known auxotrophisms, to deduce that two closely linked genes, known as the H-factor, control heterokaryon formation in *Rhizoctonia solani* AG4; differences in one or both genes allow the growth of the heterokaryotic secondary mycelia. AG1 is believed to have a similar system of mating (Anderson, 1982) meaning that the mating systems are primarily heterothallic (outbreeding). AG8 is also believed to be primarily heterothallic (Yang *et al.,* 1992). Evidence of homothallism has also been observed in the laboratory within AG1 (Julian *et al.,* 1997) and AG4 (Adams and Butler, 1982) when primary hyphae produced basidia. This phenomenon is known as haploid fruiting or monokaryotic fruiting and has been observed in other basidiomycetes (Julian *et al.,* 1997). However, far fewer spores were observed in haploid fruiting within AG1 (as much as a 500-fold difference), suggesting that the role this has to play

under field conditions maybe limited. Also, recent genetic analysis with restriction fragment length polymorphism (RFLP) markers of the Texan AG1-IA rice population has found high levels of gene flow, suggesting active heterothallism (Rosewich *et al.,* 1999).

1.1.3 Hyphal interactions in *Rhizoctonia solani*

The mating genetics of AG2 and AG3 are believed to be different from other AGs; they do not produce tufts and are presumed to be homothallic and predominantly clonal in nature (Cubeta and Vilgalys, 1997). The mating genetics of the remaining AGs are yet to be studied in detail. Hyphal interactions are not only limited to opposing primary mycelia; heterokaryon-homokaryon mating (known as the Buller phenomenon) has been observed in the laboratory (Cubeta *et al.,* 1993; Julian *et al.,* 1996; Yang *et al.,* 1994) but may have little relevance in natural populations, particularly as the saprophytic capability of single basidiospore isolates is usually limited (Cubeta and Vilgalys, 1997).

Perhaps the most common interaction between *R. solani* isolates is the vegetative compatibility interaction or anastomosis between two heterokaryotic mycelia. Vegetative incompatibility is also known as somatic, heterokaryon or mycelial incompatibility. In most basidiomycetes, vegetative and sexual compatibility are both regulated by the same genes (Leslie, 1993) but these mechanisms have been shown to be independent in at least one AG of *R. solani* (AG1) (Julian *et al.,* 1996). Both sexual and vegetative anastomosis involves the breakdown of two

hyphal walls and the union of two separate plasma membranes allowing cytoplasmic continuity and the transfer of nuclei and organelles.

Hyphal fusions are essential within the colony of a higher fungus for efficient functioning. These hyphal fusions allow the individual colony to become an interconnected hyphal network facilitating the transport of nutrient and signalling molecules to anywhere in the colony. To operate this mechanism the fungus must possess the machinery necessary to target and recognise other hyphae. Observations have supported the hypothesis that a diffusible attractant is the initial stimulus for anastomosis in *Rhizoctonia* (McCabe *et al.,* 1999). In a normal system, hyphae avoid each other to ensure a radiating, explorative hyphal network, however anastomosis requires the opposite, and this may be initiated by environmental factors, most likely nutrients. In *Rhizoctonia*, the amount of available nutrients, especially nitrogen is known to affect anastomosis (Carling, 1996).

Despite the benefit of an efficient mycelial network, considerable risks to the colony are associated with hyphal fusion. These risks can include a loss of genetic identity and the transfer of deleterious genetic elements such as plasmids, organelles and viruses (Hoekstra, 2001). Therefore, vegetative incompatibility mechanisms are thought to exist to minimise these risks: anastomosing hyphae are usually similar if not identical. Fusion of hyphae from different vegetative compatibility groups (VCGs) results with internalised cell death, involving the closure of septal pores to isolate dying hyphal cells and cell degradation. Vegetatively compatible hyphae fuse perfectly, with hyphae from different

colonies effectively becoming one colony. In some fungi, including *Rhizoctonia,* it is possible for the colony to be a 'genetic mosaic' with multiple nuclear and perhaps mitochondrial types present (Adams, 1996). The existence of VCGs decreases the ability of an individual to generate genetic diversity and form hyphal networks, however it also halts the spread of deleterious genetic content throughout all individuals of the species and VCGs are therefore considered to increase total species fitness.

1.1.4 Anastomosis groups of *Rhizoctonia solani*

The presence of VCGs early in the lineage of *R. solani*, as a mechanism of genetic isolation, may have led to the development of the anastomosis groups seen today. AGs are not merely VCGs but a higher taxonomic unit that can contain multiple subgroups, which in turn have multiple VCGs. At least 13 AGs have been shown to exist (Carling *et al.,* 2002a) that have different life histories and differences in DNA sequence (Kuninaga *et al.,* 1997). In some classifications, some individual AGs are classed as species and given individual names (Table 1.1). AGs occupy different habitats (i.e. hosts) and often geographic locations as well as having genetic differences. Classification of individual AGs to species level has not generally been accepted. AGs, as genetically delimited and non-interbreeding groups can be defined as a biological species (i.e. they are a whole population which are actively/potentially inter-fertile). However, current taxonomy is largely based on the morphological differences and consequently, despite the lack of inter-fertility and good

molecular genetic evidence, the AGs within *R. solani* have not been classed as separate species (Roberts, 1999).

Table 1.1 Anastomosis groups of *Rhizoctonia solani*, host, associated valid names and persons credited with discovery of the AG (adapted from Roberts, 1999)

AG	Typical hosts	Associated names	Discovery credited to
1	Rice, corn, bean	*T. sasakii* (AG1-IA) *T. microsclerotia* (AG1-IB)	Parmeter *et al.* (1969)*
2	Crucifers, sugar beet, carrot,		Parmeter *et al.* (1969)*
3	Potato, tobacco		Parmeter *et al.* (1969)*
4	Bean, cereals, root rots	*T. praticola*	Parmeter *et al.* (1969)*
5	Potato, turf grass, root rots		Ogoshi, (1976)
6	Orchid mycorrhizal		Kuninaga *et al.* (1978)
7	Carnation, radish, soybean, saprophyte		Homma *et al.* (1983)
8	Cereals		Neate *et al.* (1985)
9	Crucifers, potatoes, saprophyte		Carling *et al.* (1987)
10	Wheat, barley		Ogoshi *et al.* (1990)
11	Lupin, wheat		Carling *et al.* (1994)
12	Orchid mycorrhizal		Carling *et al.* (1999a)
13	Cotton		Carling *et al.* (1999b)

*Presence of four anastomosis groups before Parmeter *et al.* (1969), who is credited with designation of AG1 to AG4.

R. solani, with its various AGs, is therefore often referred to as a 'species complex' (Cubeta and Vilaglys, 1997). A feature of the species complex, which is not common in basidiomycetes, is the display of what Burnett (2003) classed as 'complex somatic incompatibility' where a range of, often unique, reactions occurs. A range of anastomosis reactions was observed in what is perhaps the earliest work of this kind, using hyphal anastomosis reactions to differentiate strains of *Rhizoctonia* (Matsumoto *et al.,* 1932). Several different systems have been used to categorise the range of reactions present within the complex (Table 1.2).

Table 1.2 Previous categorisation systems for anastomosis reactions (adapted from Carling, 1996)

Present Class (Carling *et al.*, 1988)	**Matsumoto *et al.* (1932)**	**Flentje and Stretton (1964)**	**Parmeter *et al.* (1969)**
C0	No reaction	NR (No reaction)	0
C1	Contact	WF (Wall fusion)	1
C2	Imperfect	K (Killing)	2-Imperfect
C3	Perfect	S (Self)	2-Perfect

The system used widely today for categorising hyphal fusion, and that forms the basis of assigning isolates to AG, is that originating from the work of Carling and co-workers (Carling and Leiner, 1987; Carling *et al.,* 1988). This system consists of four categories of reaction (C0 to C3). Perfect fusion of the hyphae is classed as C3 and usually occurs between genetically identical or near identical isolates (Cubeta and Vilgalys, 1997). These isolates are therefore part of the same VCG. 'Killing' or C2 reactions are designated when hyphal wall and membrane fusion are evident, but after a period of time cell death occurs in the fused and adjacent cells. Both C2 and C3 are usually indicative of the same AG. However, C1 reactions, designated when hyphal wall contact occurs but there is no evidence of membrane fusion (Carling *et al.,* 1987), can also occur between diverse members of an AG (e.g. between subgroups) or closely related AGs (bridging relationships). Bridging relationships are known to occur between members of AG2, 3, 6, 8, 10 and 11 as illustrated in Figure 1.1 (Sneh *et al.*, 1991; Carling *et al.*, 1994; Carling *et al.*, 2002a). C0 is designated when 'no recognition' or 'no interaction' occurs between isolates meaning that the isolates are likely to belong to different AGs (Carling and Leiner, 1987).

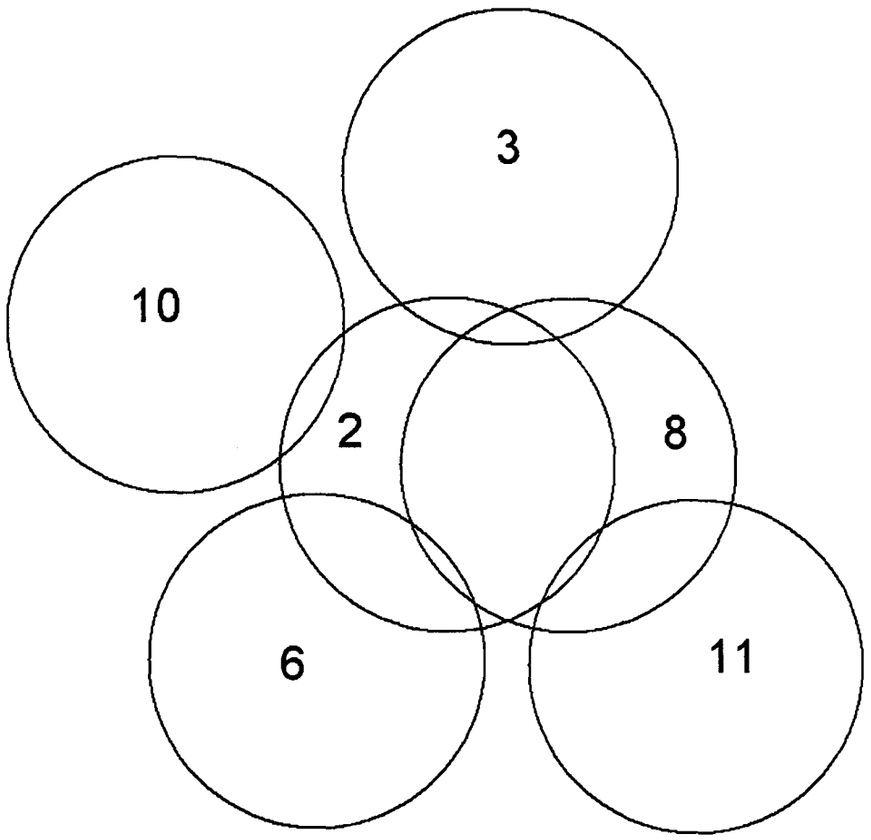


Figure 1.1 Bridging relationships in *Rhizoctonia solani* AGs (figure does not indicate intensity of bridging reaction but only to indicate currently known existing inter-AG relationships)

Figure 1.2 illustrates the complex somatic incompatibility reactions between isolates within the *R. solani* species complex. From this diagram it can be suggested that, particularly in the case of C0 reactions, recognition events occur, as hyphae are actively avoiding each other, either by negative tropisms, arrested growth when in proximity, or continued growth when in contact. Therefore, it is possible that C0 reactions could be observed between isolates of the same AG, when the colonies are immature and in the 'exploratory phase' - hence not needing to consolidate the mycelial network by establishing cross-links for efficient function. Environmental factors such as nutrient supply and life cycle phase (i.e. genetic state) can profoundly affect AG testing. Currently, several methods for the identification of AG by observation of hyphal fusions exist, including pairing isolates on water agar (Ogoshi, 1976), on cellophane

overlaying agar (Parmeter *et al.*, 1969) and on glass slides (Kronland and Stanghellini, 1988). Whichever method is selected must be used throughout the study, as differences in nutrient availability and/or environment exist between the different methods and this can affect fusion frequency.

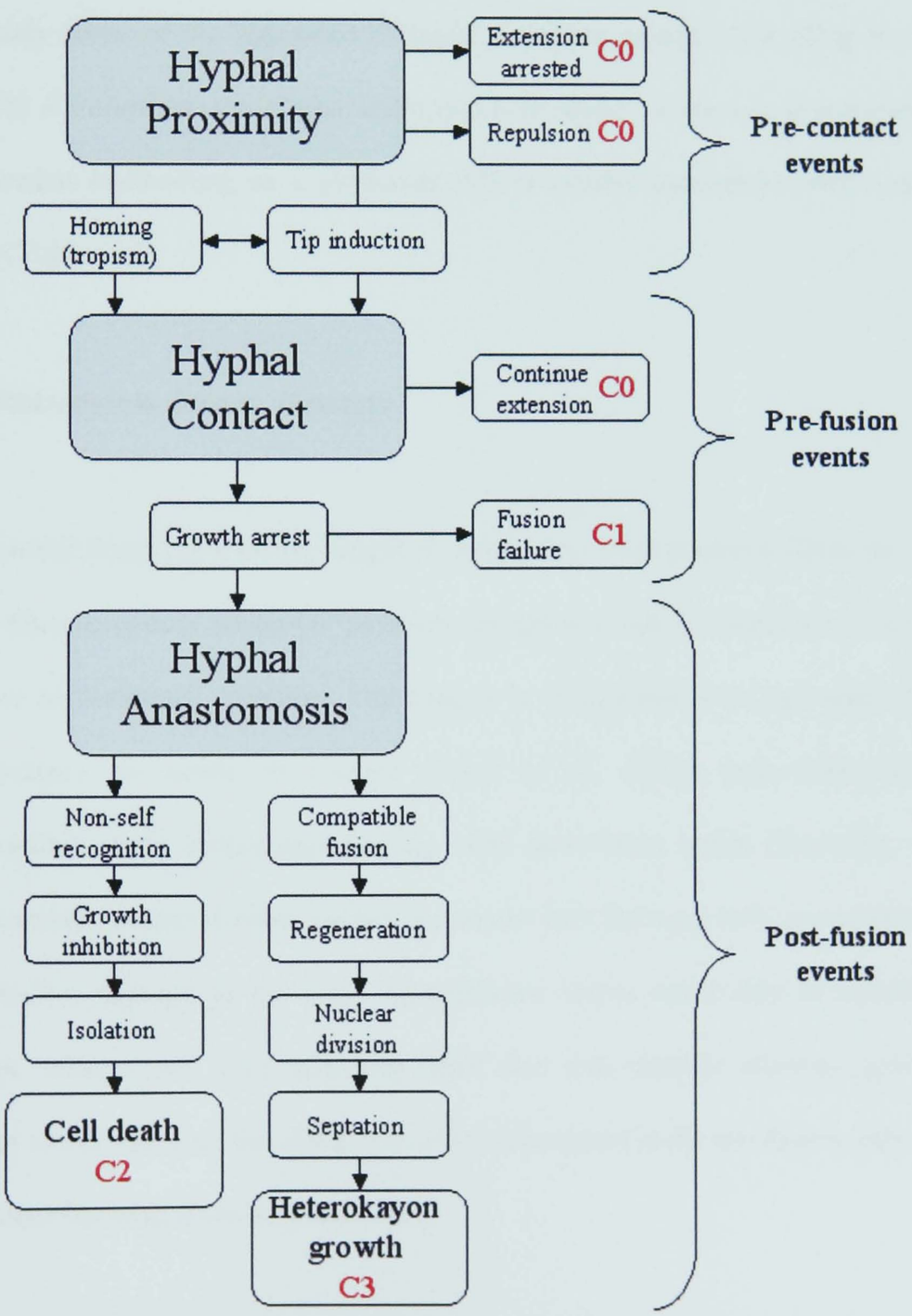


Figure 1.2 Flow diagram of the major steps in complex somatic incompatibility reactions and corresponding category within the *Rhizoctonia solani* species complex (adapted and redrawn from Glass *et al.*, 2000; Moore and Novak Frazer, 2002)

Despite such drawbacks in the identification of AG, the concept of AG still represents the single most important advance in the understanding of genetic diversity within *Rhizoctonia* (Cubeta and Vilgalys, 1997). Consequently, a multitude of AG typing assays based on immunological and molecular genetic techniques have been developed to identify AG. Such techniques have been used to study some of the important diseases *R. solani* causes. According to Adams (1988) *R. solani* has an 'almost unlimited host range'. However, this statement is somewhat misleading, as a particular AG is usually associated with a specific host (Table 1.1).

1.2 Rhizoctonia disease of potato

The initial description of the fungus *R. solani* was from potatoes (Roberts, 1999). The disease occurs wherever potatoes are grown, but is suspected to be more severe in temperate climates. The fungus is recognised as a pathogen of main importance on potato in Europe (Jeger *et al.,* 1996) and observations of marketable yield losses approaching 30% have been noted (Banville, 1989). Rhizoctonia potato disease causes economic loss through both quantitative and qualitative damage to the crop. Quantitative losses occur due to infection of stems, stolons and roots affecting tuber size and number whereas qualitative losses occur because the disease is often associated with misshapen tubers and the tuber blemish disease, black scurf.

1.2.1 Rhizoctonia potato disease symptoms and disease cycle

Figure 1.3 illustrates the disease cycle for rhizoctonia potato disease. Initially, infection of the developing stem occurs prior to emergence. Infection can arise as a result of infected seed or infested soil. Fungal contact with the developing stem results in the formation of infection cushions and penetration into the plant occurs from these cushions either with hyphae forcing their way between the epidermal cell walls or through the formation of penetration pegs (Chand *et al.*, 1985). The fungus can also enter through stomata but this is considered a rare occurrence (Dodman and Flentje, 1970).

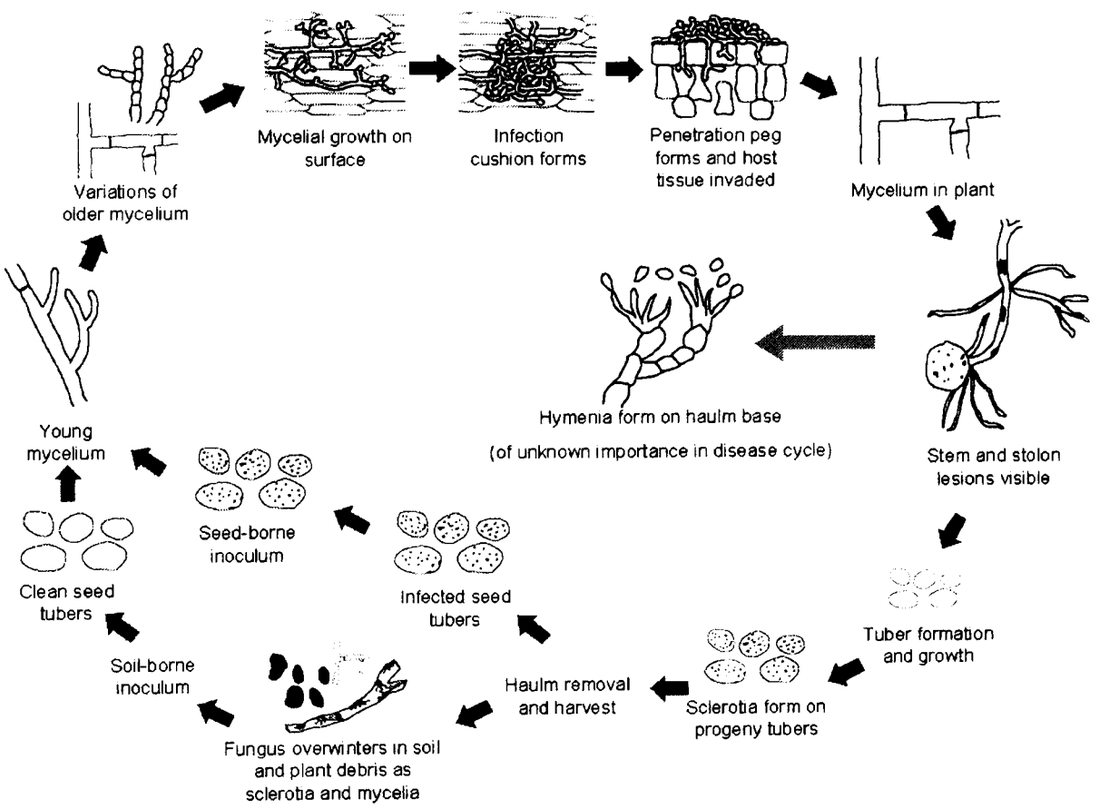


Figure 1.3 The disease cycle of rhizoctonia potato disease

Stem infection can result in the appearance of a lesion or lesions, often known as stem canker (Figure 1.4). In severe infections, lesions occupy the whole

circumference of the stem resulting in girdling and, in advanced infections, pruning. Pruning of the developing sprouts forces the development of numerous secondary stems (Baker, 1970), thereby delaying or in some cases totally preventing emergence. In severe cases of disease, infections frequently occur on secondary stems and stolons and a high incidence of stolon canker can result in particularly bad economic losses (Gudmestad *et al.,* 1999). Necrosis has been attributed to derivatives of phenyl-acetic acid elicited by *R. solani* (Frank and Francis, 1976). Isolate virulence in *R. solani* AG3 was observed to correlate with the ability to produce phenyl-acetic acid derivatives *in vitro* (Tavantzis *et al.,* 1989), indicating the importance of the phytotoxin in disease development.

Figure 1.4 Lesions on the main stem of potato caused by *Rhizoctonia solani*

Symptoms observed above ground are likely to be secondary responses due to stem infection. Leaf rolling, stunting, rosetting and purple pigmentation in the leaves have all been observed (Banville *et al.,* 1996). Purple pigmentation is attributed to anthocyanin accumulation (Baker, 1970). Late in the growing season 'white-collar' symptoms are often observed at the base of stems (Figure 1.5), particularly during moist and warm conditions (Jeger *et al.,* 1996). Basidia are typically present within the white-collar, however the significance of basidiospores in causing potato disease is uncertain: germination of these field-grown basidiospores is difficult to induce *in vitro* and their appearance coincides with canopy closure in the potato crop which would limit wind dispersal (Jeger *et al.,* 1996).

Figure 1.5 Potato stems displaying 'white-collar' symptoms

Stem and stolon canker consistently cause a decrease in tuber yields (Banville, 1989; Carling *et al.,* 1989; Hide *et al.,* 1989a) and can cause a greater number of

non-target seed sizes (Simons and Gilligan, 1997a). Yield quality is decreased by the development of sclerotia on progeny tubers, known as black scurf. Sclerotia are irregularly shaped masses of fungal mycelium brown to black in colour adhering tightly to the surface of the tuber (Figure 1.6). Sclerotia formation does not result in physical damage to the tuber but sclerotia present on seed tubers can initiate disease in subsequent crops. Few sclerotia are present prior to haulm destruction or plant senescence (Spencer and Fox, 1979; Otrysko *et al.,* 1988). Exudates from progeny tubers, in addition to volatile products from decomposing roots and stolons can stimulate sclerotial formation (Dijst, 1990).

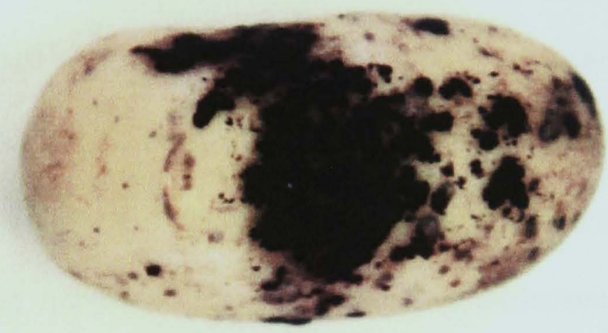


Figure 1.6 Potato tuber displaying black scurf symptoms

In some cases, rhizoctonia potato disease is associated with misshapen tubers, growth cracks (Banville *et al.,* 1996; Campion *et al.,* 2003) and tuber greening (Hide *et al.,* 1989b). Morphological alterations of the progeny tubers have been attributed to the plant's inability to process the excess photosynthate that accumulates when the flow is interrupted by stem and stolon lesions (Hartill, 1989). Netted scab symptoms (russeted scabby areas on the tuber surface) have also been observed (Baker, 1970; Back, 2003). Netted scab is thought to be the

result of a point of necrosis on the stolon tip expanding with tuber growth (Back, 2003).

1.2.2 Factors affecting disease development

Inoculum type, soil type, soil moisture and temperature can affect disease development, in addition to the isolates ability to cause infection (virulence). Seed-tuber borne inoculum of *R. solani* is often considered to be of primary importance compared to soil borne inoculum (Frank and Leach, 1980; Hide *et al.,* 1973; Weinhold *et al.,* 1982). Proximity of the seed-borne sclerotia to the emerging sprouts is thought to lead to consistently severe infections. More recent work has highlighted the importance of soil-borne infection (Hide *et al.,* 1985; Hill and Anderson, 1989; Kyritsis and Wale, 2004). Gudmested *et al.* (1999) stated that the economic significance of stolon infections was greater than of stem infection, and soil-borne inoculum has been observed to cause a higher incidence of stolon infection than with seed inoculum (Hide *et al.,* 1985). Also, a greater incidence of black scurf was observed with soil-borne inoculum than with seed-borne inoculum (Hide *et al.,* 1985). Disease development may therefore be affected by inoculum source, with seed-borne sources associated with severe stem cankers and soil-borne inoculum associated with infection of secondary stems, stolons and roots.

Disease severity can also be influenced by soil type (Hill and Anderson, 1989; Jager and Velvis, 1983); this influence was attributed to the increased presence of antagonists in certain soils (Jager and Velvis, 1983). The moisture content of

soil can also influence disease development. Kyritsis and Wale (2002a) in controlled environment studies, found that stem canker was most severe when the water holding capacity (WHC) of soil was 40% compared to 20% or 60%. This is concordant with the work of Hide and Firmager (1989) who found that stem canker severity was greater at 45% WHC compared to 75% or 90% in controlled environment conditions. Black scurf development is also influenced by moisture: black scurf severity was greater at 20% or 40% WHC than at 60% (Kyritsis and Wale, 2002a). Observations from fieldwork support these conclusions, where irrigated plants displayed less stem canker (Simons and Gilligan, 1997b).

The effect of soil temperature on disease development has been investigated largely through experiments conducted in controlled growth cabinets: temperatures of 10 to 15°C seem to be optimal for disease development (Bolkan *et al.,* 1974; Kyritsis and Wale, 2002a; Hide and Firmager, 1989; Carling and Leiner, 1990). Bolkan *et al.* (1974) found that whilst temperature influenced disease severity with low amounts of inoculum, no influence was observed when higher amounts of inoculum were used. This suggests that the role of temperature is of secondary importance to inoculum concentration. Carling *et al.,* (1990) observed that temperature could affect AGs differently, AG3 causing more stem damage at 10°C compared to 15.5 and 21°C, whilst AG5 only causing damage at 15.5 or 20°C.

AGs can differ in their relative virulence to potato, AG3 is usually associated with potato disease and is able to cause severe infection. However, several other

AGs have been shown to infect potatoes (Balali *et al.*, 1995; Bains and Bisht, 1995; Campion *et al.*, 2003; Carling and Leiner, 1990). Within an AG, isolates can differ in virulence (Tavantzis *et al.*, 1989) either due to the presence of biological variation or the presence of double stranded RNA elements in the cytoplasm, which can confer hypovirulence (Tavantzis *et al.*, 2002).

Life cycle stage can also affect virulence; isolates of AG3 originating from single basidiospore cultures are less virulent than heterokaryotic isolates (Hill and Anderson, 1989). Homokaryons of AG8 have also been observed to be less virulent to wheat than their heterokaryotic parents (Yang *et al.*, 1994).

1.3 The management of rhizoctonia potato disease

1.3.1 Host resistance

Breeding for resistance to rhizoctonia disease in potato has only met with limited success. No cultivars display complete resistance to potato. In experiments with both European (Chand and Logan, 1982; Little *et al.*, 1988 and Scholte, 1989) and North American cultivars (Leach and Web, 1993) no significant differences in susceptibility to stem canker were observed. Bains *et al.* (2002) and Kyritsis and Wale (2002b) both found differences in several cultivars for susceptibility to black scurf, however none were completely resistant to the disease. Campion *et al.* (2003) found no significant differences in susceptibility to black scurf caused by AG3. Resistance to stem canker and black scurf is present in several wild *Solanum* species (Wastie, 1994) and crosses with these wild cultivars has led to

the conclusion that resistance to *Rhizoctonia* is under polygenic control and recessive (Wastie, 1994).

Despite this success, breeding for resistance to *Rhizoctonia* is considered extremely difficult due to the presence of two phases of the disease (black scurf and stem canker), the significant affect of environmental and soil conditions in disease development (Leach and Web, 1993) and the diversity of the pathogen (Wastie, 1994). This coupled with the limited availability of resistant germplasm (Panella and Ruppel, 1996) has led to the search for methods using genetically modified (GM) plants to enhance resistance.

Such strategies involve the introduction of genes encoding for proteins able to inhibit the growth of fungi. Proteins being investigated for such a use include chitinases, β-1-3-glucanses and ribosome inactivating proteins (RIP) (Cornelissen *et al.,* 1996). Chitinase proteins sourced from other plants have conferred enhanced resistance to *R. solani* in tobacco (Broglie *et al.,* 1991), *Nicotiana sylvestris* (Vierheilig *et al.,* 1993), canola (Benhamou *et al.,* 1993) and rice (Lin *et al.,* 1995). Transgenic potato plants (cv. Désireé) showed complete resistance when modified with a chitinase gene originating from *Trichoderma harzianum* (Lorito *et al.,* 1998).

Strategies involving β-1-3-glucanases and RIPs have been investigated less intensively and showed that tobacco plants expressing barley glucanases or RIP genes displayed enhanced resistance to *R. solani* (Jach *et al.,* 1995). However, when each of these genes was co-expressed with the chitinase gene, antifungal

activity was greatly increased, possibly due to a synergistic activity of the two gene products. Recently, antimicrobial peptides have been discovered which have high *in vitro* lytic activity against an AG1 isolate of *R. solani* (Oard *et al.*, 2004). These authors have suggested that such peptides could be utilised in molecular breeding to develop resistance to the pathogen. Despite such advances, GM strategies remain largely unused due to the technology being relatively new and current public opinion against the use of the technology in Western Europe.

1.3.2 Biocontrol

Bacteria, fungi and mycophagous soil fauna have all been investigated for use as potential biocontrol agents. Strategies utilising bacteria include the use of antagonistic *Serratia*, *Bacillus* (Ciampi *et al.*, 1999) and *Pseudomonas* species (Crowe and Olseen, 2001; Duffy, 2000; Kataria *et al.*, 2002). Antibiotic producing species of *Streptomyces* have also been utilised: several species of the genus have been incorporated into the soil to control *Rhizoctonia* on a wide range of crops (Kulik, 1996). The antibiotic valadamycin A, has also been isolated from *Streptomyces hygroscopicus* and has been used to control black scurf and rice sheath blight (Kulik, 1996). Recently, fungichromin produced by *Streptomyces padanus* was observed to have a strong activity against *R. solani* AG4 *in vitro,* and successfully reduced the incidence of damping off of cabbage (Shih *et al.*, 2003). The efficacy of using such bacterial control agents may be further enhanced through bioencapsulation of the bacterial cells (Ciampi *et al.*, 1999).

The mode of antagonism of some *Streptomyces* species are antagonistic through their chitinolytic activity which can hydrolyse the cell wall of *Rhizoctonia*, Chitinolytic activity is shared by many fungal biocontrol agents of *Rhizoctonia* including *Trichoderma* and *Gliocladium* species which may also express β-1-3-glucanases and possibly also have a mycoparasitic activity. The diketopiperazine antibiotic, gliotoxin, has been isolated from *Gliocladium virens* and has been shown to have a fungistatic to fungicidal action to isolates of *R. solani*. However some anastomosis groups were shown to have a degree of insensitivity to gliotoxin (Jones and Pettit, 1987).

Beagle-Ristaino and Papavizas (1985), found that *Trichoderma* species when applied as dusts to seed potato or soil resulted in a significant reduction in rhizoctonia disease incidence. Tsror *et al.* (2001) found that *Trichoderma harzianum* was not as effective at reducing black scurf when applied to the soil surface compared to its efficacy when applied as an in furrow treatment, suggesting that the point of application is important.

Wicks *et al.* (1995) observed that *Bacillus* and *Trichoderma* species were largely ineffective as seed treatments for rhizoctonia potato disease but successes have been reported with other potential *Rhizoctonia* controlling fungi. Carisse *et al.* (2001) reported that a strain of the genus *Microsphaeropsis* could reduce AG3 sclerotial germination by over 75% *in vitro*. The *Microsphaeropsis* strain also reduced sclerotial coverage on tubers incubated at 4°C from 1.6 to 0.5 sclerotia per cm^2 whilst with formaldehyde and untreated tubers an increase in sclerotia was reported, with coverage rising from 1.2 to 7.8 sclerotia per cm^2. Similar *in*

vitro results have been reported with *Stachybotrys elagans*, which at temperatures between 14 and 20°C reduced the amount of viable sclerotia by 79% compared to the control (Benyagoub *et al.*, 1994).

The most intensively studied *Rhizoctonia* controlling fungus is the mycoparasite, *Verticillium biguttatum.* Wickes *et al.* (1995) reported that when applied to tubers as either dip or spray it could reduce the viable sclerotia population by over 90%. Application of *V. biguttatum* on seed tubers prior to planting significantly reduced grading loss and incidence of black scurf (Jager *et al.*, 1991) and has shown potential for use in conjunction with conventional chemical seed treatments to control rhizoctonia potato disease (van den Boogert and Luttikholt, 2004).

Non-pathogenic *Rhizoctonia* isolates may also act as an elicitor of the plant defence response (Jabaji-Hare *et al.*, 1999). For example, non-pathogenic binucleate *Rhizoctonia* significantly reduced the incidence of infected tubers in field experiments (Tsror *et al.*, 1999). It is also likely that such non-pathogenic isolates may be antagonistic to virulent *Rhizoctonia* by competing for nutrients available saprotrophically.

In addition to micro-organisms acting as potential biocontrol agents, mycophagous soil mesofauna have been observed to reduce the severity of *Rhizoctonia* infection. The springtail *Folsomia fimetaria* and the nematode *Aphelenchus avenae* reduced stem infection in potato individually and may have a synergistic activity when used together (Lootsma and Scholte, 1997).

1.3.3 Crop rotation

Relatively high levels of *R. solani* suppressive mycophagous soil fauna and microbes to may be achieved through crop rotation strategies. Crop rotation has ancient origins and not only reduces disease initiated by soil borne pathogens but maintains soil structure and reduces soil erosion.

Currently, no single rotation sequence exists for *R. solani* control, however a review of the literature suggests that, for disease control, potatoes should not precede potatoes (Banville *et al.,* 1996). Long rotations are considered better than short rotations: increased disease severity was observed in crops grown in short rotations (Read *et al.,* 1995) and Peters *et al.,* (2003) found significantly less disease in three year, as opposed to two year, rotations. Carling *et al.*, (1986a) indicated that five-year rotations are more appropriate as they were able to detect *R. solani* in soil after two years but not after five years. Scholte (1992) suggested that the cropping frequency of potatoes and not the role other crops play is the overriding factor. This is not universally agreed, other studies have reported that preceding potatoes with solanaceous crops (Banville *et al.,* 1996), sugar beet (Banville *et al.,* 1996), clover (Celletti *et al.,* 1990; Johnston *et al.,* 1994), oats (Leach *et al.,* 1993; Sprecht and Leach, 1987; van Elsas *et al.,* 2002) or millet (Sprecht and Leach, 1987) can increase disease incidence. In contrast, preceding potato crops with buckwheat (Sprecht and Leach, 1987; Frank and Murphy, 1977), grass (van Elsas *et al.,* 2002) or broccoli (Leach *et al.,* 1993) reduced disease incidence.

Sprecht and Leach (1987) found that the total population of *Rhizoctonia* species increased after buckwheat crops, the reduction in disease incidence after buckwheat therefore could be explained in that much of this total *Rhizoctonia* population was composed of non-pathogenic strains. Leach *et al.* (1993) hypothesised that broccoli reduced disease incidence by changing the physical structure of the soil, with its taproot, to be suppressive to *R. solani*. However many of these studies were carried out with only AG3 or *R. solani* isolates unassigned to AG and other AGs pathogenic to potato may be affected differently by rotation sequences (Petkowski and de Boer, 2001).

In addition to rotation the use of cover crops has also been investigated. The use of fodder rape as a cover crop has been shown to significantly reduce the incidence of black scurf in field trials (Little *et al.,* 2004). The reduction was attributed to the presence of biocidal isothiocyanates associated with the decomposition of fodder rape tissue. However, this adds significant costs, as cover crops need to be sown, mulched and incorporated into the soil over the six months prior to planting.

1.3.4 Cultural disease control

Tillage practices may influence populations of soil-borne *Rhizoctonia.* Some studies suggest that these practices have an even greater influence disease severity than rotation. Leach *et al.* (1993) observed that chisel ploughing significantly reduced rhizoctonia disease compared to mouldboard ploughing. Chisel ploughing was thought to place crop residues near the soil surface, where

as mouldboard ploughing deposited crop residues deep in the soil and hence possible inoculum was close to the planted seed tuber. Chisel board ploughing, leaves a crop residue near the soil surface, where it is mixed through the top layers of the soil, which is likely to increase the residue decomposition rate, consequently encouraging the growth of microbes that inhibit *Rhizoctonia*. Peters *et al.* (2003) provided evidence to support this when they found that the root zone bacteria most effective at inhibiting *R. solani* were recovered more from minimum tillage soils as compared to soils with conventional tillage. Such minimum tillage practices also have the benefit of increasing soil organic matter content, improving soil hydraulic properties and offering increased protection from soil erosion (Peters *et al.*, 2003).

Tillage can also affect the presence of weeds in a crop and weeds may influence the population of *Rhizoctonia* in the soil. Isolates of *R. solani* have been obtained from some weed species present in potato fields (Jager *et al.*, 1982; Sturz *et al.*, 1995; Carling *et al.* 1986b) but evidence suggests that these isolates have an epiphytic relationship as opposed to a pathogenic relationship with such plants. However, it may be possible that weeds can facilitate survival of *R. solani* between potato crops as the pathogen may gain nutrients through root exudation or by existing saprotrophically on dead weed plants.

Practices favouring rapid emergence may reduce the incidence and/or severity of stem canker infection as mature stems express greater resistance to infection (Hide *et al.*, 1985). Planting tubers in warmer (>8°C) soil, despite this being closer to the optimum growth temperature of *R. solani*, decreases stem canker

severity as the plant can reach maturity faster (Hide and Firmager, 1989). A reduction in planting depth has also been employed, but this may be detrimental to the root system and thus cause problems in a dry growing season (Banville *et al.,* 1996).

Planting clean seed can reduce the incidence of disease (if no soil-borne inoculum is present), since just 5% surface coverage of sclerotia on the tuber can initiate disease in cooler climates (Banville and Carling, 2001). Samples of seed could be visually checked for black scurf and eye plug tests may be performed to detect hyphae in an otherwise symptomless seed tuber (Hide *et al.,* 1968). Mulder *et al.* (1992) investigated the use of green crop harvesting to produce clean seed for planting based on the observation that immature tubers lack black scurf symptoms. The disadvantage of this was that the immature tubers lacked storability due to their susceptibility to skinning, bruising and secondary rots.

1.3.5 Chemical control

Fungicides are the most widely used method for the control of rhizoctonia diseases (Kataria and Gisi, 1996), and have been successfully employed to combat many rhizoctonia diseases. Chemicals are applied either to the aerial plant parts, seed or the soil, (the latter two only are applicable to rhizoctonia potato disease). A wide range of chemicals representing several fungicide groups have been used to control the various diseases caused by *Rhizoctonia* including: aromatic hydrocarbon, carboxamide, benzimidazole, dicarboximide, triazole, morpholine, phenylurea, phenylpyrrole and strobilurin groups. Other chemicals

including boric acid, calcium oxide, sodium hypochlorite, formaldehyde and the antibiotic validamycin A have also been used. Detailed information on the effectiveness of specific fungicides to *Rhizoctonia* species is available in the reviews by Kataria and Gisi (1996; 1999).

A considerable range of chemicals (listed in Table 1.3) have been used to combat rhizoctonia potato disease. Cross comparisons between studies are difficult due to the differences between individual studies; including location, inoculum type, disease assessments and fungicide rate. Another problem is that many studies do not consider the AG of the inoculum used. Differences exist in the sensitivity of individual AGs to certain fungicides. For example, with pencycuron, a widely used seed tuber treatment in the UK, isolates of AG5, AG7 and AG8 show a high degree of insensitivity to the fungicide in tests *in vitro* (Kataria and Gisi, 1999).

In the UK, seed treatments are predominantly used to control rhizoctonia potato diseases. Presently, popular seed treatments include pencycuron and flutolanil. However, seed treatments are only effective against seed-borne inoculum and do not give protection against soil-borne inoculum. Recent studies have suggested that the fungicide azoxystrobin, which may be suitable for soil application, provides at least some control of soil-borne rhizoctonia disease (Hilton *et al.*, 2004; Wale *et al.*, 2004).

Table 1.3 Studies investigating the effectiveness of chemical control of rhizoctonia potato disease

Active ingredient (a.i)	Effect*	Application	Trial type	Reference
Calcium oxide	+	Seed	Field	Bains *et al.* (2002)
Fludioxonil	+			
Captan	+			
Iprodione	+			
Thiabendazole	+			
Thiophanate-methyl	+			
Mancozeb	+			
Difenoconazole	+			
Pencycuron	+	Seed	Field	Bradshaw and Thomas (1992)
Tolclofos-methyl	+			
Flutolanil	+			
Formaldehyde	+	Seed	Field	Carling *et al*, (1989)
Pentachloronitrobenzene (PCNB)	+	Soil	Field	Davis and Groskop (1979)
Sodium hypochlorite	+	Seed	Field	Errampalli and Johnston (2001)
Thiophanate-methyl	+			
Thiabenzidole	+	Seed and soil	Field	Hide and Cayley (1982)
Iprodione	+			
Benodanil	+			
Maneb	+	Seed		
Azoxystrobin	+	Soil	Field	Hilton *et al.* (2004); Wale *et al.* (2004)
Thiabendazole	+	Seed	Field	Platt *et al.* (1993)
Fludioxonil	+	Seed	Field	Du Plessis and Meyer, (1998)
Iprodione + imazalil	+			
Mepronil	+			
Pencycuron	+			
Thiabendazole	+			
Tolcolofos-methyl + thiram	+			
Iprodione	+	Seed	Field	Rahkonen *et al.* (2002)
Mancozeb	+			
Flutolanil	+			
Pencycuron	+	Seed	Glass house and Field	Thind *et al.* (2002)
Methoxy ethyl mercury bromide	+			
Carboxin	+			
Carbendazim	+			
Carboxin + Thiram	+			
Boric acid	+			
Edifenphos	-			
Tolclofos-methyl	+	Soil	Field	Tsror *et al.* (1999)
Flutolanil	+			
Iprodione	+			
fenpiclonil	+	Soil and seed		
Pencycuron	+			
Azoxystrobin	+	Soil	Field	Virgen-Calleros *et al.* (2000)
Pencycuron	+			
Fluazinam	+			
Captan	-	Seed	Field	Weinhold *et al.* (1982)
Benomyl	+			
Formaldehyde	+			
Pencycuron	+	Seed	Sclerotia germination	Wicks *et al.* (1995)
Formaldehyde	+			
Fenpiclonil	+			
Tolclofos-methyl	+			
Sodium hypochlorite	-			
Metham sodium	+	Soil	Field	Wicks *et al.* (1996)
Pencycyuron	+	Seed		
Iprodione	+			
Tolcofos-methyl	+			
Formaldehyde	+			

*+, chemical displayed at least some control in at least one trial; -, chemical displayed no significant effect in reducing disease

The application of agrochemicals other than fungicides to potato crops to control other potentially detrimental organisms may also influence the populations of soil-borne *Rhizoctonia.* For example, the herbicides fluometuron and prometryn have been shown to suppress the pathogen in soil (Beam *et al.,* 1977) and the herbicide pendimethalin was shown to decrease hyphal growth *in vitro* (Harikrishnan and Yang 2001).

In addition, insecticides have also shown the potential to reduce rhizoctonia infection (Kataria and Gisi, 1996). Organophosphorus insecticides, particularly aromatic phosphorothioates are inhibitors of the cutinase released by the infection structures of the fungus. Conversely, some carbamate insecticides have been shown to increase rhizoctonia disease severity; carbofuran, aldicarb and cloethocarb in oil seed rape crops (Kataria and Verma, 1993) and lindane in potatoes (Hofman *et al.,* 1991). Therefore it is suggested that their use should be avoided where rhizoctonia disease is a problem (Kataria and Gisi, 1996).

Nematicides have been found to have variable effects on rhizoctonia potato disease development. The nematicides oxamyl and ethoprophos have been shown to have fungitoxic effect in laboratory trials (Hofman *et al.,* 1991) and a reduction in stolon cankers and black scurf incidence in pots treated with aldicarb, fosthiozate and oxamyl was observed in glasshouse trials (Back *et al.,* 2002). In the field, Back *et al.* (2002) observed a reduction in stolon canker in potatoes in soil treated with aldicarb but this was not statistically significant. In field trials conducted by Hide and Read (1991), oxamyl was applied to the soil over a period of several years. In four of the years, a reduction of black scurf was

observed in oxamyl treated plots. However, stem canker severity was greater than untreated plots in one year. Scholte (1987) found that stem canker infection was consistently higher in plots treated with aldicarb. In addition, Ruppel and Hecker, (1982) found increased rhizoctonia root rot of sugar beet in soils treated with aldicarb.

Hoffman *et al.* (1991) investigated why *R. solani* infection sometimes increased when soil was treated with nematicides and observed lower numbers of mycophagous nematodes, mites and springtails in nematicide treated fields. In laboratory trials, a number of these mesofauna species were found to actively feed on *R. solani* mycelium. This may also explain the increase in *R. solani* infections following carbamate insecticide applications. In some conditions it is apparent that the application of nematicides can aggravate soil-borne infection, however, it may be possible to take advantage of the fungitoxic activity of nematicides as part of a wider disease management programme.

1.4 Aims of this study

Anecdotal evidence has suggested that the incidence and severity of rhizoctonia potato disease has increased significantly in recent years (Dr. P. Jenkinson pers. comm.), which may have resulted in crops of lower quality and yield. Limited data from surveys on the incidence of potato diseases have confirmed this observation: between 1963 and 1976 the incidence of black scurf in surveyed seed tuber stocks from England and Wales averaged 24% (Hide, 1981), whilst between 1996 and 2000 the equivalent figure was 60% (Bradshaw *et al.*, 2002).

No historical data is available for the incidence of stem canker, however between 1996 and 2000 stem canker was present in 72% of the potato crops surveyed (Bradshaw *et al.,* 2002). This data indicates that *Rhizoctonia* is an important pathogen in potato crops and since severe infections are able to cause marketable yield losses approaching 30% (Banville, 1989), any increase in the severity or incidence of the disease may cause significant economic losses.

The increase in incidence and severity of the disease may be explained by environmental factors, particular growing practices or changes in the population of the causal agent, *R. solani*. Such population changes could include one or more of the following:

1. Outside introduction of isolates more aggressive to potato into Britain
2. Recombination or hybridisation events producing more aggressive isolates
3. A diverse population of *R. solani* in potato crops consisting of several AGs
4. Isolates developing resistance to fungicides
5. Gradual increase of the total British soil population of *R. solani*

Presently several aspects of rhizoctonia potato disease in Britain are not fully understood, such as the lack of a clear relationship between the severity of stem canker and the severity of black scurf later in the season. For example, some crops display low levels of black scurf when severe stem canker was observed earlier in the season. In addition, no data on the relative incidence of individual AGs present in potato crops in Great Britain exists, since the presence of AGs

that do not express typical symptomology may explain the lack of a clear relationship between stem and tuber diseases. Few studies have compared rhizoctonia infection of potato caused by different AGs, those that have were not conducted in Britain and were limited to glasshouse (Stack *et al.,* 1999) or controlled environment studies (Carling and Leiner, 1990). Also, differences in sensivity to certain fungicides are known to occur between AGs (Kataria and Gisi, 1999). It is speculated that use of such selective fungicides may cause changes in the population of *R. solani*, perhaps selecting for pathogenic AGs and the proportion of fungicide insensitive isolates present, decreasing the effectiveness of the fungicide.

To investigate this, the following plan of study was undertaken: -

- To determine if different AGs are causing potato disease in Britain, isolates of *R. solani* were collected from potato crops and tested for anastomosis group.
- The implications of different AGs causing potato disease were investigated through field and glasshouse experiments, which compared disease development between different AGs.
- DNA sequencing of representative *R. solani* isolates enabled phylogenetic relationships between isolates and subgroups to be inferred.
- From the DNA sequence data, nucleic acid detection assays were designed to detect specific AGs *in vitro* and in plant and soil samples.
- Isolates were subjected to more detailed characterisation including DNA sequence analysis, pathogenicity tests, fungicide sensitivity and physiological comparisons between isolates (growth rate, sclerotia

production and presence of thiamine auxotrophisms). This characterisation will provide an insight into the diversity and differences of isolates within and between AGs, enabling conclusions about the structure of the population of *R. solani* in potatoes.

2. General methods

2.1 Supply of chemicals and media

All chemicals used in this study were supplied by Sigma-Aldrich (Sigma, Poole, UK) unless otherwise stated. Media for microbial growth was supplied by LabM (Bury, Lancs, UK).

2.2 Maintenance and storage of fungal cultures

All aseptic operations were performed in a laminar flow cabinet. Glassware, microbial growth media and sterile distilled water (SDW) were autoclaved at 121°C and 103.4 KPa for 20 minutes. Isolates of *Rhizoctonia solani* were maintained on Petri-dishes containing potato dextrose agar (PDA). Colonies were regularly checked both macroscopically and microscopically to confirm they were pure isolates of *R. solani*. Long-term storage of the isolates was on PDA slopes at –20°C.

2.3 Assembly of isolate collection

Isolates used throughout this study are listed in Table 2.1. Isolates were assembled from the existing collections at Harper Adams University College and the Scottish Crop Research Institute (SCRI), Invergowrie. Selected isolates discovered during the course of this study were added to the collection.

Table 2.1 Isolates of *Rhizoctonia solani* used in this study

Code	Anastomosis Group	Original Host	Country of Origin	Year of isolation	Source
R58	1-IA	Paddy Rice	Japan	1999	SCRI
R59	1-IA	Unknown	Unknown	1999	SCRI
R60	1-IB	Sugar Beet	Japan	1999	SCRI
R61	1-IB	Sugar Beet	Japan	1999	SCRI
R62	1-IC	Sugar Beet	Japan	1999	SCRI
R7	2-1	Potato	UK	1995	SCRI
R22	2-1	Unknown	USA	Unknown	SCRI
R42	2-1	Cauliflower	Netherlands	Unknown	SCRI
X1	2-1	Potato	UK	2001	Chapter 3
X46	2-1	Potato	UK	2001	Chapter 3
X52	2-1	Potato	UK	2001	Chapter 3
X81	2-1	Potato	UK	2001	Chapter 3
Y2	2-1	Potato	UK	2002	Chapter 3
Y3	2-1	Potato	UK	2002	Chapter 3
Y25	2-1	Potato	UK	2002	Chapter 3
Y63	2-1	Potato	UK	2002	Chapter 3
Z1	2-1	Potato	UK	2002	Chapter 3
R23	2-2	Peanut	Japan	Unknown	SCRI
R38	2-2	Sugar Beet	Netherlands	1999	SCRI
R68	2-2IIIB	Pea	Japan	1999	SCRI
R70	2-2IIIB	Unknown	Unknown	1999	SCRI
R71	2-2IV	Sugar Beet	Japan	1968	SCRI
R72	2-2IV	Soil	Unknown	1971	SCRI
R73	2-2IV	Sugar Beet	Japan	1970	SCRI
R74	2-2IV	Sugar Beet	Japan	1973	SCRI
R75	2-2IV	Sugar Beet	Japan	1975	SCRI
R78	2-3	Soybean	Japan	1991	SCRI
R98	2-BI	Soil	Japan	1977	SCRI
R1	3PT	Potato	UK	1995	SCRI
R36	3PT	Potato	UK	1998	SCRI
X22	3PT	Potato	UK	2001	Chapter 3
X34	3PT	Potato	UK	2001	Chapter 3
X40	3PT	Potato	UK	2001	Chapter 3
X56	3PT	Potato	UK	2001	Chapter 3
X72	3PT	Potato	UK	2001	Chapter 3
Y29	3PT	Potato	UK	2002	Chapter 3
UN	3PT	Potato	UK	2001	Harper Adams
I2	3PT	Potato	UK	2000	Harper Adams
I3	3PT	Potato	UK	2000	Harper Adams
I5	3PT	Potato	UK	2000	Harper Adams
R25	4	Bean	UK	Unknown	SCRI
R51	4	Unknown	USA	Unknown	SCRI
R86	4	Sugar Beet	Japan	1962	SCRI
R87	4	Soil	Japan	1962	SCRI
R89	4	Sugar Beet	Japan	1972	SCRI
R90	4	Sugar Beet	Japan	1973	SCRI
R48	5	Potato	France	Unknown	SCRI
R52	5	Soybean	Japan	Unknown	SCRI
T1	5	Couch grass	UK	2002	Chapter 3
Y55	5	Potato	UK	2002	Chapter 3
R26	6	Soil	Japan	Unknown	SCRI
R54	6	Soil	Japan	Unknown	SCRI
R97	7	Soil	Japan	1979	SCRI
R28	8	Barley	UK	Unknown	SCRI

2.4 Isolation of *Rhizoctonia* from potato samples

Plant samples were washed under running tap water. Individual sclerotia from tubers were removed using a scalpel and placed onto streptomycin amended Tap Water Agar (TWA; 15 g Agar No.2, 0.125 g streptomycin sulphate l^{-1}). Sections of diseased tissue from stems, stolons and roots (3-5 mm) were surface sterilised in sodium hypochlorite (1% available chlorine) for at least one minute, rinsed in two changes of SDW and allowed to dry before being placed onto streptomycin amended TWA. After 48-72 h incubation at 18°C, hyphal tips from colonies of *Rhizoctonia* were identified under a dissecting microscope and transferred on to PDA.

2.5 Hyphal fusion assays

A modified version of the clean slide technique (Kronland and Stanghellini, 1988) was used to observe hyphal fusion (Figure 2.1). Isolates under test were grown on PDA at 20°C until the agar was fully colonised, 8 mm plugs were then removed and placed on a clean glass slide 20 – 30 mm apart from an 8 mm plug of the other test isolate. The glass slide was then placed in a moisture chamber. Moisture chambers consisted of four 50 x 50 mm^2 pieces of paper towel moistened with 2 ml of SDW lining a Petri dish. The moisture chambers with slides were then incubated at 20°C until hyphae of the confronted isolates overlapped. The area of hyphal overlap was observed at 40X magnification to search for potential fusion events, which were then confirmed at higher magnification (100X and 400X).

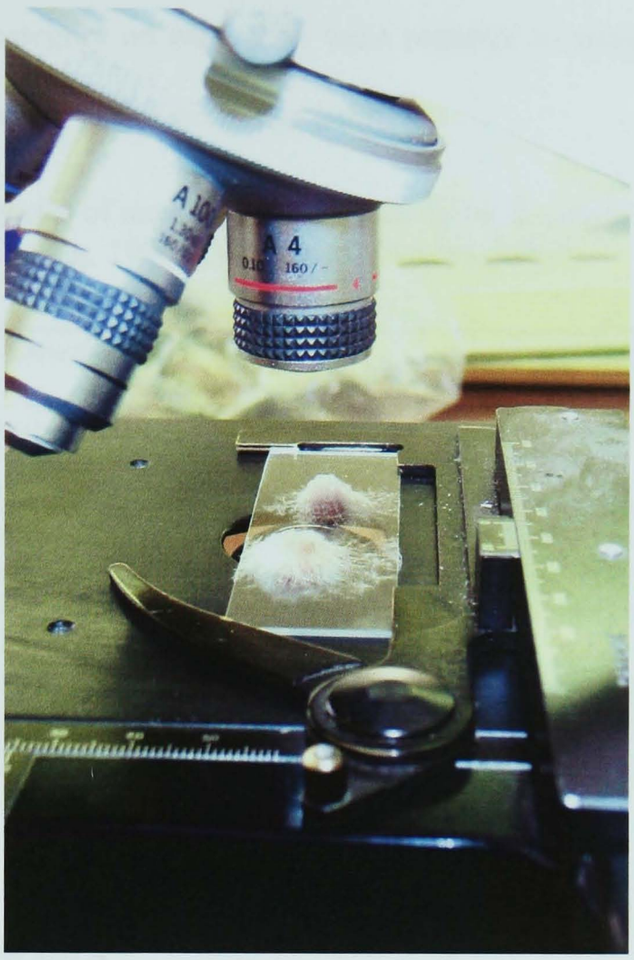


Figure 2.1 Observation of hyphal fusion using the clean slide technique

Nuclei were stained using the safranin O technique (Bandoni, 1979) to determine the nuclear state of individual isolates and to aid observation of hyphal fusion. Placing one drop of alkaline safranin solution with a separate drop of 3% potassium hydroxide stained the area of interest in the hyphal network. The safranin solution consisted of 6 ml of 0.5% (w/v) safaranin O solution, 10 ml 3% (w/v) potassium hydroxide solution, 5 ml glycerine and 79 ml SDW.

Individual hyphal fusion reactions were assigned to one of the four categories in Table 2.2. Each pairing was replicated three times. Three or more reactions

in the highest category on each slide were required to assign the interaction and determine anastomosis groupings (AG).

Table 2.2 Categories of anastomosis reaction in the *R. solani* species complex (adapted from Carling, 1996; Cubeta and Vilgalys, 1997)

Category	Description of hyphal interaction	Relationship	
C0	No recognition observed	Different anastomosis group (AG)	*Increasing genetic relatedness* ↓
C1	Hyphal wall contact only, no evidence of membrane contact	Different AG or diverse members of the same AG	
C2	Hyphal wall and membrane fusion evident, death occurring in fused and adjacent cells	Same AG but different vegetative compatible population (VCG)	
C3	Hyphal wall and membrane fusion with no evidence of cell death (perfect fusion)	Same AG and same VCG, possibly clonal	

2.6 Extraction of DNA

DNA was extracted from mycelia harvested from 4 day old colonies using a Puregene® Genomic DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Total DNA was quantified by spectrophotometry (Model: DU640, Beckman Instruments Inc. Fullerton, USA); the Warburg-Christian coefficient (Equation 2.1) was used to determine DNA concentration and Equation 2.2 was used to determine DNA purity.

Equation 2.1

$$[\text{Nucleic acid in ng } \mu l^{-1}] = (-36 \times (A_{280}-A_{328}))+((62.9 \times (A_{260}-A_{328}))$$

Equation 2.2

$$\text{Purity} = (A_{260}-A_{328})/(A_{280}-A_{328})$$

Where A is absorbance of ultraviolet light at the wavelength denoted in subscript.

For pure DNA the ratio generated by Equation 2.2 is 1.8, impure DNA samples (ratios below 1.7 and above 2.2) were not used. DNA was diluted to 1 ng μl^{-1} for diagnostic PCR.

2.7 Polymerase chain reaction (PCR) and electrophoresis of PCR products

All PCR amplifications were based on a standard set of conditions, annealing temperature and primers pairs were changed accordingly. PCR conditions were: initial denaturation at 94°C for 75 s, followed by 35 cycles of 94°C for 15 s, annealing temperature for 15 s and 72°C for 45 s, the final cycle having an extra 4 min 15 s at 72°C. PCR mixtures (25 µl) consisted of 100 µM of each nucleotide, 20 U ml^{-1} of Red Hot Taq polymerase (ABgene, Epsom, Surrey, UK), 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg ml^{-1} gelatin, 0.5 mg ml^{-1} Tween 20, 0.5 mg ml^{-1} Nonidet P-40, 5 ng of sample DNA and appropriate primers at 100 nM. Primers were synthesised by MWG Biotech AG (Ebersberg, Germany).

Ten µl of the PCR products were electrophoresed through 2% (w/v) agarose gels (containing 0.5 µg ml^{-1} ethidium bromide) in TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.0). The size of PCR products was determined using a gel documentation system (Gel Doc 1000, BioRad Laboratories Ltd, Hemel Hempstead, UK) and ØX174 *Hind*II DNA (ABgene) as the size standard.

2.8 Production and bulking of inoculum

Inoculum was prepared using a method adapted from Papavizas and Davey (1962). Isolates were removed from storage and grown on 9-cm Petri-dishes of PDA for four days at 20°C. Fifteen 8 mm diameter plugs were taken from a culture of each isolate and were used to inoculate plastic bags containing 3 kg of autoclaved sand-cornmeal media (100 parts sand, 15 parts distilled water and five parts cornmeal). Bags were thoroughly mixed, fastened at the neck (though not airtight) and left at room temperature until the sand-cornmeal media was fully colonised (approximately four weeks). Bags were then opened, checked for any sign of contamination by bacteria or fungi and contaminated bags were discarded. The remaining bags were emptied into large containers for each treatment and thoroughly mixed.

2.9 Disease assessment of seed potato tubers

Potato seed batches used in pathogenicity tests, field and glasshouse trials were initially tested for disease to ensure they did not have high levels of

disease that may have affected the experiments. Fifty seed tubers were selected from each batch to be used and washed to remove soil and debris before being inspected macroscopically for a range of diseases including black scurf, silver scurf, powdery scab and signs of rotting caused by bacteria.

Seed was also inspected microscopically using the 'eye plug' test as adapted from Hide *et al.,* (1968). Eye plugs of 5 mm diameter and 15 mm depth were taken from the tuber using a cork borer and scalpel. The plug was then transferred to a moisture chamber and incubated for seven days at 15°C, after which the plugs were examined microscopically (30X magnification) for signs of infection. Diseases could be identified using the descriptions of Hide *et al.* (1968). Seed batches containing signs of potentially damaging diseases were not used.

2.10 Rhizoctonia disease assessment of potato plants

The key of Carling and Leiner (1990) was used to assess stem disease where:

0 = no damage or lesions present

1 = minor damage, one to several lesions less than 5 mm in size

2 = moderate damage, lesions larger than 5 mm and some girdling present

3 = major damage, large lesions and girdling or death present on most stems

4 = all stems killed

Root disease was assessed as follows:

0 = no visible infection

1 = 1 to 10% of the root area infected

2 = 11 to 25% of the root area infected

3 = 26 to 50% of the root area infected

4 = 51% or more of the root area infected

In field trials, tuber yield and black scurf disease incidence and severity were assessed on tubers at each of the size grades: 65 to 85 mm, 45 to 65 and under 45 mm. Prior to assessment, tubers were rinsed in water to make sclerotia more conspicuous. Black scurf was scored for all harvested tubers as follows:

0 = no sclerotia present

1 = less than 1% of the tuber surface area covered in sclerotia

2 = 1 to 10% of the tuber surface area covered in sclerotia

3 = 11 to20% of the tuber surface area covered in sclerotia

4 = 21to 50% of the tuber surface area covered in sclerotia

5 = 51% or more of the tuber surface area covered in sclerotia

2.11 Preparation of Micrographs

A Leitz florescence photo-microscope (DM-RMB) connected to a video camera (Photonic Science Ltd.) was used for the preparation of micrographs. Images recorded using Image Proplus version 4.5 (Media Cybernetics Inc., Silverspring, MD, USA).

2.12 Statistical analysis

All statistical analysis unless mentioned otherwise was undertaken with Genstat (version 6.2.0.235, 2002), Lawes Agricultural Trust, VSN International Limited, Hemel Hempstead, UK.

3. Anastomosis groups of *Rhizoctonia* isolates collected from British potato crops between 2001 and 2003

3.1 Introduction

Several anastomosis groups of *Rhizoctonia solani* are able to cause disease on potato (Banville *et al.*, 1996). Other *Rhizoctonia* species are believed to have little or no role in causing disease on potato (Carling and Leiner, 1990). Often, anastomosis groups of *R. solani* are limited in their ability to infect different parts of the potato plant. For example, Carling *et al.* (1998) demonstrated the infection of stems, stolons and tubers but not roots by AG7, whilst Hide and Firmager (1990) observed that AG8 was only capable of infecting potato roots. Table 3.1 summarises the anastomosis groups associated with potato disease and their occurrence on the various plant organs.

Despite the capability of several anastomosis groups to infect potato, AG3 is generally acknowledged as the principle cause of rhizoctonia potato disease; 16 of the 17 investigations listed in Table 3.2 show AG3 to be the predominant group in potato crops. The exception to these findings was the work of Anguiz and Martin (1989), who reported that AG4 was the predominant group in warm, lowland areas of Peru. They showed that AG4 had an optimum growth temperature of between 25-28°C, compared to 20-25°C for AG3 and thus suggested that climate can influence the distribution of certain AGs. The presence of AG4 was also

observed in other investigations, and again the apparent influence of climate can be seen; in the partially tropical climate of Mexico, 26.4% of infections were caused by AG4 (Virgen-Calleros *et al.*, 2000), but in the cool Alaskan climate, Carling and Leiner (1986) found no AG4 present.

Table 3.1 Anastomosis groups (AG) infecting potato and organ(s) reported to be infected

AG	Stems and Stolons	Black Scurf	Roots	Reference
1		Yes[1]	Yes[2]	Abe and Tsuboki (1978)[1] Bandy and Leach (1988)[2]
2-1	Yes[3]	Yes[4]		Cedeno *et al.* (2001)[3] Chand and Logan (1983)[4]
2-2	Yes[5]	Yes[6]		Demirci and Döken (1993)[5] Abe and Tsuboki (1978)[6]
3	Yes	Yes	Yes	Numerous
4	Yes[7,8,9]	Yes[9]	Yes[9]	Anguiz and Martin, (1989)[7] Bains and Bisht (1995)[8] Balali *et al.* (1995)[9]
5	Yes	Yes	Yes	Bandy *et al.* (1984)
7	Yes	Yes	No	Carling *et al.*, (1998)
8			Yes*	Hide and Firmager (1990)
9	Yes		No	Carling *et al.* (1987)
11	Yes*		No*	Carling *et al.* (1994)
12	No*		No*	Carling *et al.* (1999a)
13	Yes*		Yes*	Carling *et al.* (2002b)

*Artificially inoculated and in controlled environment conditions only

Table 3.2. Summary of previous rhizoctonia potato disease investigations into anastomosis groups present in potatoes

Region	Climate*	% AG causing black scurf/tuber infection	Number of black scurf/tuber infection samples analysed	% AG causing stem or stolon cankers	Number of stem or stolon samples analysed	All Rhizoctonia infections (including roots & hymenial isolates)	Total analysed	Reference
Japan	C/D	96% AG3 0.7% AG1 0.4% AG2-2 3% AG5	273	N/a	N/a	N/a	N/a	Abe and Tsuboki (1978)
Peru	A	100% AG3	30	8% AG3 56% AG4 36% unknown	50	34% AG3 40% AG4 26% unknown	100	Anguiz and Martin (1989)
Alberta, Canada	D	N/a	N/a	76.6% AG3 10.9% AG4 10.9% AG5 1.6% unknown	64	N/a	N/a	Bains and Bisht (1995)
S. Australia	B/C	97.7% AG3 0.5% AG4 1.9% AG5	216	87.5% AG3 12.5% AG5	24	93.2% AG3 6.1% AG4 2.5% AG5 0.35% binucleate	279	Balali *et al.* (1995)
Maine, USA	D	100% AG3	88	80% AG3 13.8% AG5 3.1% unknown 3.1% binucleate	65	85% AG3 10.7% AG5 0.6% AG1 2.3% unknown 1.1% binucleate	168	Bandy *et al.* (1988)
Brazil	A/B	100% AG3	10	100% AG4	2	83.2% AG3 16.7% AG4	12	Bolkan and Ribiero (1985)

Region	Climate*	% AG causing black scurf/tuber infection	Number of black scurf/tuber infection samples analysed	% AG causing stem or stolon cankers	Number of stem or stolon samples analysed	All Rhizoctonia infections (including roots & hymenial isolates)	Total analysed	Reference
Spain and Morocco	C	100% AG3	26	N/a	N/a	N/a	N/a	Campion *et al.* (1999)
France	C	93.7% AG3 4.2% AG2-1 2.1% AG5	238	100% AG3	3	93.8% AG3 4.1% AG2-1 2.1% AG5	241	Campion *et al.* (2003)
Alaska, USA	D	97.3% AG3 2.7% AG2-1	73	67.6% AG3 25.4% AG2-1 2.8% unknown 4.2% binucleate	71	73.7% AG3 20.1% AG2-1 3.6% unknown 2.7% binucleate	224	Carling and Leiner (1986)
Venezuela	A	N/a	N/a	N/a	N/a	92.6% AG3 5.7% AG2-1 1.7% binucleate	176	Cedeno *et al.* (2001)
N. Ireland	C	95.6% AG3 4.4% AG2-1	182	N/a	N/a	N/a	N/a	Chand and Logan (1983)
Erzurum, Turkey	D	96.3% AG3 1.2 % AG2-2 1.2% AG4 1.2% AG5	82	2% AG2-1 2% AG2-2 81.4% AG3 8.8% AG4 5.9% AG5	102	1.09% AG2-1 1.63% AG2-2 88.0% AG3 5.5% AG4 3.8% AG5	184	Demirci and Döken, (1993)
North Dakota, USA	D	N/a	N/a	N/a	N/a	58% AG3 16% AG4 25.2% AG5 0.8% unknown	119	Gudmestad *et al.* (1989)

Region	Climate*	% AG causing black scurf/tuber infection	Number of black scurf/tuber infection samples analysed	% AG causing stem or stolon cankers	Number of stem or stolon samples analysed	All Rhizoctonia infections (including roots & hymenial isolates)	Total analysed	Reference
Denmark	C	100% AG3	60	N/a	N/a	N/a	N/a	Justesen *et al.* (2003)
Canada	D	99.3% AG3	720	N/a	N/a	N/a	N/a	Otrysko *et al.* (1985)
Victoria, Australia	C	68% AG3 11.2% AG2-1 2.4% AG2-2 1.9% AG4 3.4% AG5 13.1% other	206	74.4% AG3 23.1% AG2-1 2.5%AG2-2	39	69% AG3 13.1% AG2-1 2.4% AG2-2 1.6% AG4 2.9% AG5 other 11.0%	245	Petkowski *et al.* (2003)
Ujjain, India	A	100% AG3	10	69.2% AG3 30.1% AG4	13	84% AG3 16% AG4	25	Suresh and Mall (1982)
South Africa	B/C	99.3% AG3 0.7% AG3	411	82% AG3 13% AG4 5% AG5	39	97.8 AG3 1.1 AG4 1.1 AG5	450	Truter and Wehner (2004)
Central Mexico	A/B	N/a	N/a	N/a	N/a	73.5% AG3 26.5% AG4	68	Virgen-Calleros *et al.* (2000)

N/a = not applicable

*Climate class is based on the Köppen system (as Griffiths, 1976): A, Tropical moist; B, Dry; C, Moist mid latitude with mild winters; and D, Moist mid latitude with cold winters.

Binucleate *Rhizoctonia* and AG2-1 have been found in several surveys, but have not usually been shown to cause more than a small percentage of rhizoctonia disease cases. Similarly, AG5 has been found in several investigations, but has never accounted for more than 14% of the *Rhizoctonia* samples analysed. Gudmestad *et al.* (1989) suggested that cropping history is an important factor in determining which AGs are present. In their study, AG4 was only present in samples grown in fields with no previous potato cropping history. In addition, they found that 56% of *R. solani* isolates recovered from wheat were AG5, thereby showing that certain groups can successfully colonise other hosts. Other AGs found in such investigations include AG1 (Abe and Tsuboki, 1978) and AG2-2 (Demirci and Döken, 1993), their presence may also have been caused by their introduction and propagation in other crops.

Several studies have recovered a high proportion of isolates that could not be successfully assigned to a particular AG. For example, Anguiz and Martin (1989) failed to assign 26% of their isolations to AGs. Similarly, Bandy *et al.* (1988) failed to assign 2.3% and Carling and Leiner (1986) 3.6% of isolates. Since most investigations of this type have used the hyphal fusion method to determine AG, such difficulties can be understood. To overcome such failures, a combination of molecular and hyphal fusion methods were used in this study to determine the relative frequencies of anastomosis groups present in British potato crops.

Currently no data exists on the incidence of individual anastomosis groups in British potato crops and it has been suggested that rhizoctonia potato disease has

become more frequent and severe in Britain (Dr. P. Jenkinson pers. comm.) A comparison of survey data on the incidence of black scurf in seed tuber stocks from England and Wales between 1963-1976 (Hide, 1981) and 1996-2000 (Bradshaw *et al.,* 2002) supports this, as incidence increased from an average of 24% to 60%.

Hypotheses to explain the increase in severity and incidence of rhizoctonia potato disease have included the emergence of strains insensitive to fungicidal seed treatments or strains with greater virulence to potato. This work will determine if such AGs are present in Britain by isolating *Rhizoctonia* from potato samples exhibiting the disease. Knowledge of which AGs are present will prove invaluable in predicting disease development and the design of diagnostic tools for rhizoctonia potato disease within Great Britain.

3.2 Materials and Methods

3.2.1 Sample collection and isolation

Samples of potato plants and tubers showing typical and atypical symptoms of rhizoctonia disease were received from agronomists and growers between May 2001 and January 2003. Samples requested included potato with typical rhizoctonia potato symptoms: black scurf, white collar as well as stem, stolon and root cankers. Samples displaying atypical symptoms were also requested, including tuber deformations and plants with any unusual symptoms that may have been caused by the disease. No two samples originated from the same field. Isolation of *Rhizoctonia* from samples was performed as detailed in Chapter 2. Anastomosis group was determined by observation of hyphal fusion and by PCR (Chapter 2).

3.2.2 Determination of AG

DNA was extracted from pure cultures of *Rhizoctonia* isolated from each sample and diluted to a concentration of 1 ng μl^{-1} for use in PCR. DNA was checked to be of suitable quality for PCR by amplification with the rDNA internal transcribed spacer (ITS) primers ITS4 and ITS5 (White *et al.*, 1990) at an anneal temperature of 58°C. DNA samples that did not produce a PCR product with these primers were re-extracted and re-amplified. Specific PCR primers were used to determine whether samples belonged to AG2-1 (Carling *et al.*, 2002a) or

AG3 (as described in Chapter 6) at annealing temperatures of 64°C and 65°C respectively. PCR conditions are given in Chapter 2. Each AG specific PCR was repeated to confirm the result. One positive (target DNA of known AG) and three negative controls (water and DNA from isolates of two closely related AGs) were run with each batch of samples.

Primers designed to the rDNA intergenic spacer 1 (IGS1) region were also used. For amplification of the IGS1 region, primers LR12R (GAA CGC CTC TAA GTC AGA ATC C) located within the 28S RNA (Sequence from the Vilgalys Laboratory) and seq5S (CAG ATC AGA CGG GAT GCG GT) were used with an annealing temperature of 60°C. Primer seq5S was designed from the 5S rDNA sequence of *Rhizoctonia crocorum* (Genbank accession No. X00067). The length of the IGS1 region was determined for all isolates collected. Hyphal fusion assays were performed as described in Chapter 2 with the tester isolates listed in Table 3.3. Isolates were paired in all combinations available.

Table 3.3 Tester isolates used in hyphal fusion assays

Isolate	Anastomosis Group	Original Host	Country	Year of isolation
R22	2-1	Unknown	USA	Unknown
R42	2-1	Cauliflower	Netherlands	Unknown
UN	3PT	Potato	Shropshire, UK	2001
I3	3PT	Potato	Shropshire, UK	2000
R87	4	Soil	Japan	1962
R89	4	Sugar Beet	Japan	1972
R48	5	Potato	France	Unknown
R52	5	soybean	Japan	Unknown
R28	8	Barley	Scotland, UK	Unknown

3.2.3 Pathogenicity tests

Pathogenicity tests were performed in a glasshouse set at 15°C under lights with a 16h/8h light/dark cycle. Seed tubers of the cultivar Désireé (SE1) were checked for diseases (as described in Chapter 2). Selected disease-free tubers were disinfected by immersion in sodium hypochlorite (1% available chlorine) for 10 minutes and rinsed twice in distilled water. The eye of each selected tuber was removed using a 30 mm melon ball scoop. Each potato ball was then immersed in 1 mg l^{-1} giberellic acid solution for 1 h 15 mins to break dormancy. Potato balls were allowed to suberise for 72 hours before planting.

Potato balls were planted individually in pots (plastic cups 80 mm in height, 40 mm bottom diameter, with one five mm hole in the bottom). Potato balls were placed on approximately 20 mm of sieved (5 mm mesh) compost (John Innes No.2), sieved compost was then placed around and covering the potato ball to a depth of approximately 10 mm. Pots were then inoculated and the remainder of the pot filled with compost. Inoculum consisted of five 10 mm plugs taken from 10 day old PDA plates of 38 individual isolates. Isolates were selected for the pathogencity test on the basis of anastomosis group, geographical origin and availability. Each treatment was replicated six times.

All isolates were passaged through potato stems of cultivar Désireé just prior to the test. Plants were grown and inoculated as above, except varying amounts of

inoculum were used to ensure successful infection. *Rhizoctonia* was isolated from stem lesions as described previously (Chapter 2) and subcultured on PDA a maximum of two times before being used in the pathogenicity test.

After 5 weeks, plants were harvested, washed and assessed for disease (Chapter 2). The key of Carling and Leiner (1990) was used to assess stem disease. Genstat (version 6.2) was used to determine mean, minimum, maximum and variance for each AG for each disease assessment. Significant differences ($P<0.05$) between AGs were determined using unbalanced ANOVA.

3.3 Results

3.3.1 Relative incidence of anastomosis groups

One hundred and seventy-six samples were received in total from which 135 successful isolations of *R. solani* were obtained from stems, stolons, roots and tubers of plants. Successful isolations represented 32 varieties of potato and at least 25 counties of Great Britain, and also two isolates from Jersey. The full details of the origin of each isolate are listed in Appendix 1. DNA was successfully extracted from all the isolates as confirmed by UV spectrophotometry and PCR with primers ITS4 and ITS5. PCR with AG3PT specific primers gave a positive result with 125 samples. Of the remaining 10 isolates, nine tested positive for AG2-1. One sample (isolate Y55) did not test positive with either the AG2-1 or AG3PT specific primers and was determined to belong to AG5 through using hyphal fusion assays. Relative incidence of anastomosis groups isolated from various plant parts is given in Table 3.4.

Table 3.4 Incidence of anastomosis groups (AG) from various plant parts

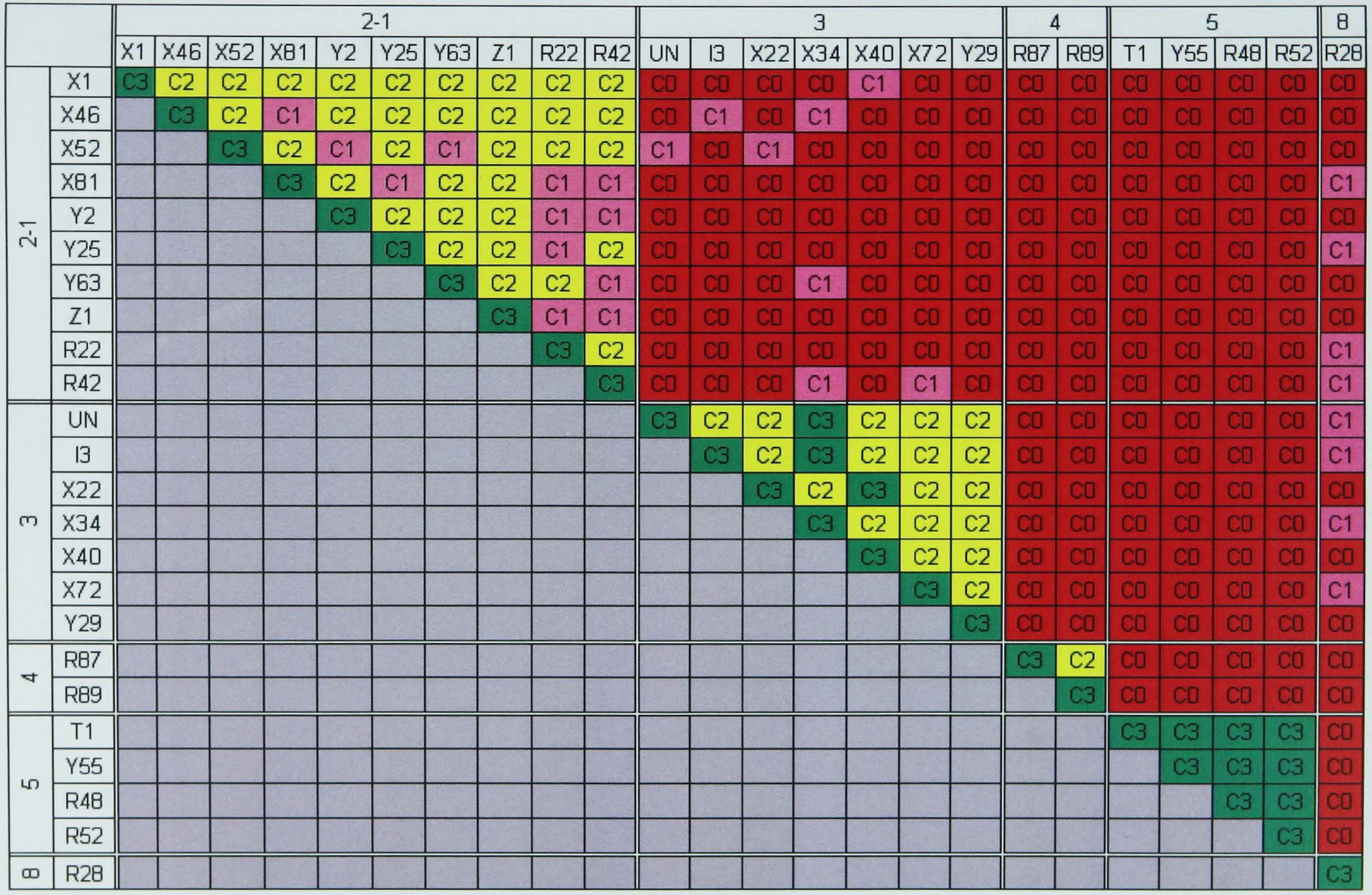
AG	Stem and Stolon Cankers	Black Scurf	Root Sclerotia	Root Lesion	Total
AG2-1	4	5	0	0	9
AG3PT	50	64	8	3	125
AG5	0	1	0	0	1
Total	54	70	8	3	135

3.3.2 Couch grass isolate

Despite only one isolate of AG5 being found causing potato disease, another isolate of AG5 was found associated with the investigation. Couch grass (*Elytrigia* [≡ *Elymus* ≡ *Agropyron*] *repens*) was present in several of the samples received, and *R. solani* was isolated from one couch grass stem base in a sample originating from Shropshire. The isolate was designated isolate T1 and assigned to AG5 through observation of hyphal fusion (Figure 3.1). Numerous isolations from the diseased potato stem within this sample were taken and all were assigned to AG3.

3.3.3 Hyphal fusion assays

All pairings are shown in Figure 3.1. Isolates were selected for pairings on the basis of anastomosis group as determined by the PCR assay, and origin. All isolates of AG2-1 found, except Y3, were tested for hyphal fusion ability. Isolates of AG3PT were selected on the basis of origin. Both the couch grass and black scurf isolates of AG5 were tested.



		2-1										3							4		5				8
		X1	X46	X52	X81	Y2	Y25	Y63	Z1	R22	R42	UN	I3	X22	X34	X40	X72	Y29	R87	R89	T1	Y55	R48	R52	R28
2-1	X1	C3	C2	C2	C2	C2	C2	C2	C2	C2	C2	C0	C0	C0	C0	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0
	X46		C3	C2	C1	C2	C2	C2	C2	C2	C2	C0	C1	C0	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0
	X52			C3	C2	C1	C2	C1	C2	C2	C2	C1	C0	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0
	X81				C3	C2	C1	C2	C2	C1	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C1
	Y2					C3	C2	C2	C2	C1	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0
	Y25						C3	C2	C2	C1	C2	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C1
	Y63							C3	C2	C2	C1	C0	C0	C0	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0
	Z1								C3	C1	C1	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0
	R22									C3	C2	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C0	C1
	R42										C3	C0	C0	C0	C1	C0	C1	C0	C0	C0	C0	C0	C0	C0	C1
3	UN											C3	C2	C2	C3	C2	C2	C2	C0	C0	C0	C0	C0	C0	C1
	I3												C3	C2	C3	C2	C2	C2	C0	C0	C0	C0	C0	C0	C1
	X22													C3	C2	C3	C2	C2	C0	C0	C0	C0	C0	C0	C0
	X34														C3	C2	C2	C2	C0	C0	C0	C0	C0	C0	C1
	X40															C3	C2	C2	C0	C0	C0	C0	C0	C0	C0
	X72																C3	C2	C0	C0	C0	C0	C0	C0	C1
	Y29																	C3	C0	C0	C0	C0	C0	C0	C0
4	R87																		C3	C2	C0	C0	C0	C0	C0
	R89																			C3	C0	C0	C0	C0	C0
5	T1																				C3	C3	C3	C3	C0
	Y55																					C3	C3	C3	C0
	R48																						C3	C3	C0
	R52																							C3	C0
8	R28																								C3

Figure 3.1 Results of pairings in hyphal fusion assays performed on 15 isolates with tester isolates listed in Table 3.3 (C0, no interaction; C1, hyphal contact; C2, killing reaction; C3, Perfect fusion)

All isolates tested were self-anastomosing, with a C3 reaction observed when isolates were paired with themselves, as shown in Figure 3.2 with isolate R22. Pairings between AG2-1 isolates resulted in C1 and C2 reactions and C2 and C3 reactions were observed in pairings amongst isolates of AG3 PT. Pairings between the AG5 isolates were all C3, and pairings between the two AG4 tester isolates were C2. Some bridging (C1) reactions were observed in some pairings between AG2-1 and AG3PT, and also between those groups and the AG8 isolate. AG4 and AG5 did not show any other class of reaction other than C0 when paired with isolates from any other AG.

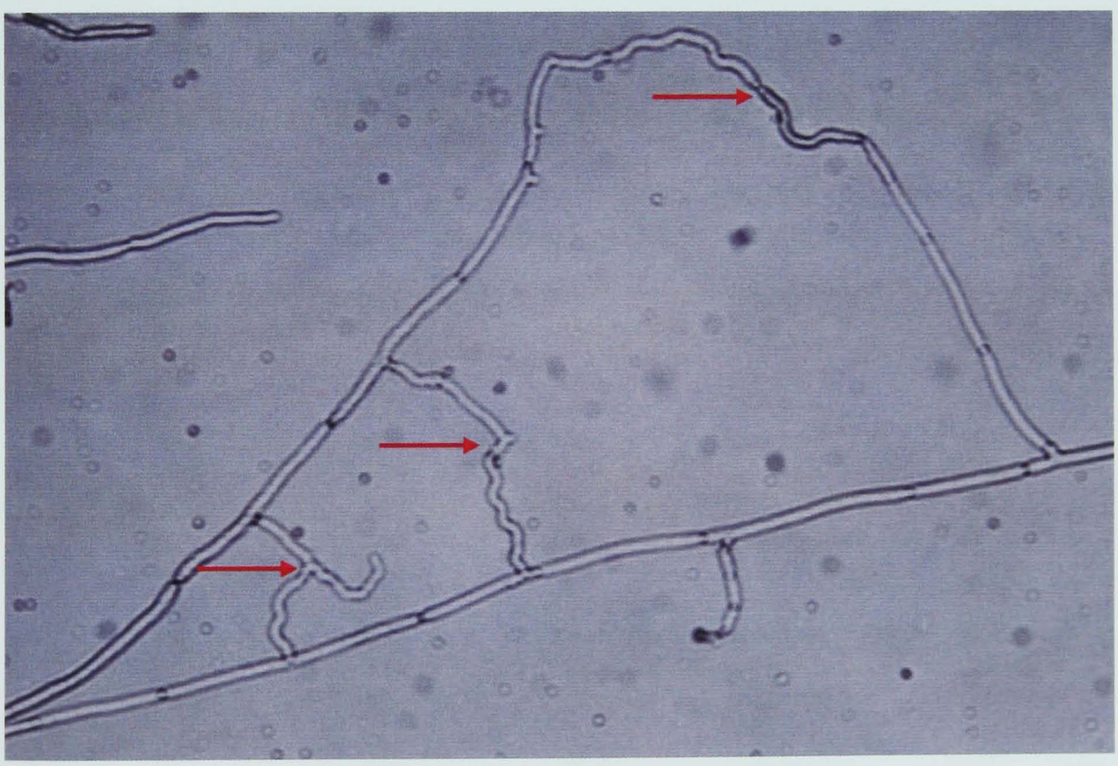


Figure 3.2 Multiple C3 reactions observed in a self-pairing of isolate R22. Arrows indicating point of fusion.

3.3.4 PCR amplification of isolates using primers designed to the rDNA IGS1 region

PCR amplification of DNA from all isolates using primers to the IGS1 region produced products of variable length (Table 3.5). All 125 isolates of AG3 from potato plus the AG3PT and AG8 tester isolates gave a PCR product of approximately 680 bp in length. All four isolates of AG5 tested gave an IGS1 product of 620 bp. Three different lengths of the IGS1 region were found amongst AG2-1 isolates. All four AG2-1 isolates with an IGS1 length of 510 bp were isolated from black scurf samples. The four isolates with an IGS1 length of 550 bp were isolated from stem or stolon canker samples. DNA obtained from AG2-1 isolate X1, which was isolated from black scurf, produced a PCR fragment of 570 bp, similar to that of the AG 2-1 tester isolates R22 and R42.

Table 3.5 Geographical and disease origin of isolates and corresponding rDNA IGS1 lengths for each anastomosis group (AG) of *R. solani*

AG	Isolate	rDNA IGS length (bp)	Disease/Host	Country/County of origin	Year of isolation
2-1	X1	570	Black Scurf	Shropshire	2001
	X46	550	Stolon	Cheshire	2001
	X52	550	Stem	Scotland	2001
	X81	510	Black Scurf	Scotland	2001
	Y2	510	Black Scurf	Shropshire	2002
	Y3	550	Stem	Lincolnshire	2002
	Y25	550	Stem	N. Yorkshire	2002
	Y63	510	Black Scurf	Scotland	2002
	Z1	510	Black Scurf	Scotland	2003
	R22	570	Unknown	USA	Unknown
	R42	570	Unknown	Netherlands	Unknown
3PT	*All isolates*	680	Various	UK	2000-2003
5	T1	620	Couch	Shropshire	2002
	Y55	620	Black Scurf	Norfolk	2002
	R48	620	Tuber lesion	France	Unknown
	R52	620	Soybean	Japan	Unknown
8	R28	680	Barley	Scotland, UK	Unknown

3.3.5 Pathogenicity tests

Results of the pathogenicity tests are shown in Table 3.6. Stem disease indices were highest on average for AG5 but this was not significantly different to the AG2-1 and AG3PT isolates. No stem disease was observed in the control (un-inoculated) plants and AG8 infected plants. Root disease was greatest for the AG8 infected plants, but this was not significantly different to AG3 infected plants.

Table 3.6 Mean, minimum, maximum and variance in stem and root disease indices and percentage stem and stolon infection and percentage stem pruning for all isolates tested by AG

		Control	AG2-1	AG3PT	AG5	AG8	lsd	sed	%cv
Number of Isolates		1	7	28	2	1			
Stem disease index score	*Mean*	0 a	1.39 b	1.78 b	1.92 b	0 a	0.35	0.35	53.39
	Min	0	0	0.25	1.2	0	0.15	0.15	
	max	0	4	3.75	3.0	0	0.50	0.50	
	Variance	0	1.50	0.63	0.33	0			
% Stem infection	*Mean*	0 a	65.05 b	84.15 c	94.58 c	0 a	18.84	9.56	30.48
	Min	0	0	20	75	0	7.98	4.05	
	max	0	100	100	100	0	26.65	13.52	
	Variance	0	1015.6	495.8	97.5	0			
% Stem pruning	*Mean*	0 ab	19.2 bc	22.2 c	1.7 a	0 ab	19.32	9.80	123.71
	Min	0	0	0	0	0	8.18	4.15	
	max	0	100	80	20	0	27.33	13.87	
	Variance	0	910.1	565.6	33.3	0			
% Stolon infection	*Mean*	0 a	5.77 a	24.49 b	28.61 b	8.33 ab	22.38	11.35	137.28
	Min	0	0	0	0	0	9.81	4.98	
	max	0	66.67	100	66.67	25	31.55	16.00	
	Variance	0	216.2	966.5	576.7	166.7			
Root disease index score	*Mean*	0 abc	0.26 bc	0.92 d	0 ab	1.17 d	0.31	0.16	52.06
	Min	0	0	0	0	1	0.13	0.07	
	max	0	2	2	0	2	0.44	0.22	
	Variance	0	0.25	0.13	0	0.17			

AGs with same letters are not significantly different (P<0.05)

In all assessments variance was greatest for the AG2-1 isolates, one AG2-1 isolate (Y25) was the most virulent of all isolates tested with an average stem disease index of 3.5, however some AG2-1 isolates only caused small lesions, resulting in low disease scores. Both AG5 isolates caused large, brown lesions consistently on potato stems but no root disease. Pathogenicity of AG5 towards couch grass was investigated by planting couch grass seeds in otherwise standard pathogenicity tests with AG5 inoculum. Brown lesions were observed on couch grass roots and stem bases for both T1 and Y55 isolates (Figure 3.3) and no signs of infection were visible in un-inoculated couch grass plants. Isolation and identification of *Rhizoctonia solani* AG5 from the inoculated couch grass plants confirmed Koch's postulates, suggesting AG5 to be pathogenic to couch grass.

Figure 3.3 Lesions present on couch grass infected with *Rhizoctonia solani* AG5 (isolate T1)

3.4 Discussion

Isolates of *R. solani* from potato plants in Britain belonged to three anastomosis groups, predominantly AG3PT with a small proportion of isolates from AG2-1 and AG5. The predominance of AG3 is expected as its predominance was reported in other countries (Table 3.2), however this is the first investigation to determine AGs occurring in potato crops in Great Britain. The relative proportions of AG2-1 and AG5 in this investigation is similar to the work of Campion *et al.* (2003) and Chand and Logan (1983). Campion and co-workers found that 94% of black scurf infections were caused by AG3 in France, AG2-1 making up 4% and AG5 2%. Chand and Logan found 96% of black scurf was AG3 and 4% AG2-1 in Northern Ireland. As France and Northern Ireland are geographically close to Britain such similarities can be expected. However work by Cedeno *et al.* (2001) in the warmer climate of Venezuela found AG3 and AG2-1 in similar proportions to this work. Perhaps this could be due to particular cropping practices, suggesting that cropping practice may be more important than the environment in governing AG distribution in some cases.

The application of molecular diagnostics in this study allowed isolates to be assigned to AG; some previous studies such as Anguiz and Martin (1989), Bains and Bisht (1995) and Bandy *et al.* (1988) were unable to assign all isolates to AG. This is probably because of the presence of new, unknown AGs and/or ambiguities in the hyphal fusion technique. Previous studies have observed

difficulty in confidently identifying AG2-1 as C1, C2 and C3 hyphal fusion categories were all present (Carling and Leiner, 1986; Schneider *et al.,* 1997a). In this study the use of the primer sets for AG2-1 (Carling *et al.,* 2002a) allowed identification of the AG. Hyphal fusion assays were categorised as C1 and C2 between AG2-1 isolates in this study, therefore AG typing solely by observation of hyphal fusion alone would have been difficult due as the C1 reaction can occur between distantly related members of the same AG or between closely related AGs (Carling *et al.,* 1988).

The presence of C1 and C2 reactions between isolates of AG2-1 indicates diversity. Diversity was also indicated by a wide range in virulence observed in the pathogenicity tests, some caused severe stem infection (the AG2-1 isolate, Y25 was more pathogenic than any other isolate tested), whilst others did not. Diversity was also indicated in the presence of three different types of rDNA IGS1 regions. Isolates could be assigned to three groups based on IGS1 type: *s* for short IGS1 type (approximately 510 bp), *n* for 'normal' (approximately 550 bp) or *l* for 'long' (approximately 570 bp). This greater genetic diversity within AG2-1 may increase the risk of resistance to fungicides and its ability to survive on other hosts compared to other groups of *R. solani.*

The length of the IGS1 region was the same for all isolates of AG3PT, as well as for AG5, indicating these groups are more homogenous. In AG3PT, less diversity was also present in the hyphal fusion reactions with C2 and C3 reactions present.

The presence of C3 suggests members of the same vegetative compatibility group are present, or possibly the isolates are clones. This is similar to the work of Kuninaga *et al.* (2000a) who observed C2 and C3 reaction between isolates of AG3. However recent studies of AG3 populations in Denmark (Justesen *et al.*, 2003) and North America (Ceresini *et al.*, 2002a) using DNA markers suggest that both clonality and limited recombination occur.

AG5 was observed also to be a homogenous group. In this investigation AG5 was found twice and only once actually infecting potato. This is the first report of AG5 in Great Britain. Its presence on couch grass may be of importance in disease control as couch grass may harbour the pathogen over winter an increase the overall soil-borne *Rhizoctonia* inoculum. AG5 was found only twice (both in Shropshire) and both isolates were of the same vegetative compatibility group (C3 reactions) suggesting that this group could survive poorly in potato fields or have a limited distribution in Great Britain.

The presence of AG8 was not detected in this investigation. AG8 has been observed to cause a barley root disease in Britain (Burton *et al.*, 1988) and cause root infection of potato plants in controlled conditions (Hide and Firmager, 1990). In the pathogenicity tests in this study, the AG8 isolate, R28, was also observed to cause severe root infections. In this study, agronomists, growers and researchers Britain were asked for samples of both typical and atypical symptoms of *R. solani*. As AG8 exists almost entirely on the roots it is difficult to notice, also

diseased roots have to be thoroughly washed to observe the lesions present. Previous studies (Table 3.2) did not detect AG8 possibly due to these reasons. Further work could involve actively taking samples of roots and soil from potato fields to determine the presence of AG8 and any other AGs not detected in this study.

The presence of several AGs in potato crops means that this should be considered in the design of diagnostic assays for rhizoctonia potato disease. An effective assay would have to be able to detect all the AGs associated with potato disease. The presence of several AGs may also be an important consideration in disease management strategies, as AGs are known to differ in their sensitivity to fungicides (Kataria and Gisi, 1999). Host range is also different between AGs; AG2-1 isolates can proliferate on a variety of hosts including brassicas, lettuce, cereals, tulips, spinach and sugar beet (Tu *et al.,* 1996) and in this study AG5 was found on couch grass. Presence of these crops or weeds in potato growing fields may increase the soil-borne inoculum present at potato planting.

4. Investigating the virulence of individual anastomosis groups of *Rhizoctonia solani* to potato

4.1. Introduction

Previous work has provided data on the incidence of AGs in potato crops in a number of countries and on specific cases of particular AGs infecting potato (Tables 3.1 and 3.2). However, relatively little work has been undertaken to compare the ability of different AGs to initiate potato disease, furthermore, this work is nearly all glasshouse and controlled environment studies.

In a comprehensive study, Carling and Leiner (1990) compared isolates from AGs 1 to 9 in controlled environment cabinets, and found significant differences in the severity of infection on potato initiated by each of the AGs. For example, AGs other than 3, 4, 5 and 8 caused minimum damage to potato stems and roots whereas AG4 and 5 only caused significant disease at temperatures above 15°C.

Balali *et al.* (1995) found similar results to Carling and Leiner (1990) in that AGs 3, 4, 5 and 8 could all cause severe potato infections. In a glasshouse study conducted at 25°C they observed deep stem cankers, girdling and pruning with AGs 3, 4 and 5 whilst only superficial stem infections with AG8. AG4 and AG8 both caused severe root infections compared to root infections caused by AG3 or AG5. Hide and Firmager (1990) found that AG8 was only capable of infecting potato roots and did not cause stem canker or black scurf.

Bains and Bisht (1995) reported that AG3 caused significantly more severe stem disease compared to AGs 4 and 5. AG2-1 was reported to rarely cause stem canker and when present, the severity of symptoms was minimal (Carling and Leiner, 1986; Carling and Leiner, 1990; Chand and Logan, 1983). However, recently Petkowksi and de Boer (2001) reported that some AG2-1 isolates could cause stem infection of a severity comparable to AG3.

The severity of black scurf on potato tubers caused by different AGs also varies. Both Hide and Firmager (1990) and Balali *et al.* (1995) observed no black scurf associated with AG8 infection in controlled environment conditions. Balali *et al.* also showed that AG4 did not cause black scurf, whereas AG5 only caused slight (1-25 sclerotia per tuber) to moderate (25-50 sclerotia) black scurf and AG3 had the ability to cause severe black scurf (50 or more sclerotia). Conversely, Campion *et al.* (2003) found that AG5 did not cause black scurf on any of the five cultivars they tested but did cause tuber deformations and superficial alterations similar to that reported by Bandy *et al.* (1984), who found that AG5 produced sunken brown lesions on the cultivar Katahdin. Campion *et al.* (2003) also reported that AG3 caused a 70-100% incidence of black scurf on tubers in pathogenicity tests and that whilst AG2-1 did not cause any black scurf, AG2-1 did have the ability to cause tuber deformations.

To date, no field experiments comparing disease development between different AGs have been reported, particularly with British isolates, many of

the studies discussed above were limited to glasshouse and controlled environment conditions. The aims of this study were to investigate rhizoctonia potato disease caused by different AGs through glasshouse and field experiments. Hyphal fusion assays and PCR techniques discussed earlier were also employed to confirm AG identity of the isolates from infected plant tissue.

4.2 Methods

4.2.1. Glasshouse experiment

For each treatment, 5 kg of the appropriate inoculum (Table 4.1) was mixed with 50 kg John Innes No. 2 compost using a cement mixer. Uninoculated pots were used as the control treatment; these were prepared by mixing silver sand instead of inoculum with compost. After mixing, approximately 5.5 kg of inoculated compost was added to each of 10 pots (height 22 cm, diameter 25 cm) and the cement mixer was washed using Virkon (Antec, Sudbury, UK), sodium hypochlorite (1.2% available chlorine) and at least two rinses of water. Unsprouted seed tubers (cv. Désirée, SE2, grade 35 to 45 mm) showing no visible signs of black scurf were then planted at a depth of 15 cm (rose end facing upwards) into the centre of each pot. Pots were then placed on benches in a glasshouse set at approximately 15°C and under lights for 16 h a day. Plants were watered as required and were harvested after 6 weeks. Harvested plants were washed and assessed for disease.

Table 4.1 Isolates used in the glasshouse experiment

AG*	Isolate	Original host	Year Isolated
2-1 'l'	X1	Potato	2001
2-1 'n'	X52	Potato	2001
2-1 's'	X81	Potato	2001
3PT	X22	Potato	2001
5	Y55	Potato	2002
8	R28	Barley	Unknown

* 's', 'n' and 'l' refers to rDNA IGS1 types designated in the previous chapter.

4.2.2. Field Experiments

Field experiments were undertaken in 2001 and 2003. For both experiments, plots were 3.66 x 5 m, consisting of two beds, each with two rows. Plots were arranged in a randomised block design. Each treatment was replicated five times. Twenty seed tubers were planted in each row 25 cm apart. Planting was carried out with the aid of a manually operated device (Figure 4.1), which allowed the tuber to be placed at a depth of approximately 15 cm. Approximately 50 g of inoculum was placed on top of the seed tuber using a measured scoop in the centre two rows. Silver sand was used instead of inoculum in control (un-inoculated) plots. Isolates used for inoculum in the 2001 and 2003 field experiments are shown in Table 4.2. Isolates used in the 2003 field experiment were passaged through potato stems prior to use. Plots were raked even after planting. Crop husbandry practices are shown for each field experiment in appendices 1 and 2.

Figure 4.1 Planting and inoculation of seed tubers

Table 4.2 Isolates used in each field experiment

AG tested	Isolate used	Original host	Year isolated
2001			
2-1 'n'*	R7	Potato	1995
3PT	R1	Potato	1995
3PT	R36	Potato	1998
3PT	UN	Potato	2001
4	R25	Unknown	1989
2003			
2-1 's'*	X81	Potato	2001
3PT	Y29	Potato	2002
5	T1	Couch Grass	2002
2001 and 2003			
8	R28	Barley	Unknown

*Refers to IGS1 length.

4.2.2.1 Agronomy of the growing crop

In both field experiments the percentage of emerged plants was determined daily from 18 to 26 days after planting. Once all plants had emerged, ground cover assessments were performed on a weekly basis until approximately 7 weeks post planting. Ground cover assessments to monitor canopy development were done with the aid of a grid containing one hundred 91.5 by 65 mm subdivisions. This was placed over each bed as in Figure 4.2 and the presence of part of the canopy in more than 50% of each division was scored. Haulm height was measured for ten plants per plot selected at random, prior to the first harvest.

Figure 4.2 Ground cover grid to measure canopy development

4.2.2.2 Harvest and disease assessment

For each plot, starting at 75 cm from one end, five complete plants were taken by hand from each centre row for stem, stolon and root disease assessments at approximately seven weeks after planting. Plants were kept in a cold store until they were assessed. At harvest, a further five plants were hand lifted from the opposite end of the plot. Disease assessments were performed as described in Chapter 2 except the disease index used in 2001 was from Back (2003). To fulfil Koch's postulates, ten cultures of *R. solani* were isolated from each plot where possible at each harvest and AG identity confirmed using PCR and hyphal fusion assays.

4.2.3. Statistical analysis

Least significant differences (l.s.d) at the 95% level were determined using Genstat (version 6.2). Any data that was not normally distributed was arcsine transformed before analysis.

4.3 Results

4.3.1 Glasshouse experiment

Percentage infection and pruning of stems and stolons, and stem root disease indices are given in Table 4.3. Over 80% of main stems were infected except in the un-inoculated pots (0%) and those inoculated with AG2-1 'l' (44.3%) and AG8 (3.3%). All main stems were infected in pots inoculated with AG5, however, only a low percentage of pruning occurred in these pots, unlike AG2-1 'n' inoculated pots, where a fifth of all main stems were pruned. Secondary stem infection was observed in pots inoculated with AG2-1 'n' and AG3 and secondary stem pruning was observed with AG2-1 'n' infected plants, however this secondary stem infection and pruning was not significant, possibly due to the fact that few secondary stems were produced in pots inoculated with other isolates, making comparisons difficult.

Table 4.3 Percentage of main stems, secondary stems, total stems and stolons infected and pruned, and stem and root disease indices as measured in a glasshouse experiment

	AG2-1 'l'	AG2-1 'n'	AG2-1 's'	AG3 PT	AG5	AG8	Control	l.s.d	s.e.d	%cv
% Main stems infected	44.3 (41.6) b	86.8 (77.7) a	88.3 (78.4) a	96.7 (86.5) a	100.0 (90.0) a	3.3 (3.5) c	0.0 (0.0) c	(13.38)	(6.70)	(27.8)
% Main stems pruned	2.5 (3.0) b	20.7 (21.3) a	0.0 (0.0) b	5.8 (6.5) b	3.3 (3.5) b	0.0 (0.0) b	0.0 (0.0) b	(9.46)	(4.74)	(215.6)
% Secondary stems infected	0.0 (0.0) a	15.0 (13.5) a	0.0 (0.0) a	10.0 (9.0) a	0.0 (0.0) a	0.0 (0.0) a	0.0 (0.0) a	(14.06)	(7.04)	(489.4)
% Secondary stems pruned	0.0 (0.0) b	13.3 (12.5) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	(9..93)	(4.97)	(620.8)
% Total infection	44.3 (41.6) c	85.0 (76.5) b	88.3 (78.4) ab	97.5 (87.0) ab	100.0 (90.0) a	3.3 (3.5) d	0.0 (0.0) d	(13.33)	(6.67)	(27.7)
% Total pruning	2.5 (3.0) b	20.2 (21.1) a	0.0 (0.0) b	5.0 (6.0) b	3.3 (3.5) b	0.0 (0.0) b	0.0 (0.0) b	(9.17)	(4.59)	(213.6)
% Stolons infected	8.7 (8.1) c	33.2 (34.5) b	3.8 (5.0) c	62.2 (52.7) a	53.4 (47.0) a	0.0 (0.0) c	0.0 (0.0) c	(9.49)	(4.75)	(50.4)
% Stolons pruned	3.3 (3.5) c	12.4 (13.2) b	0.0 (0.0) c	33.7 (34.7) a	11.2 (16.1) b	0.0 (0.0) c	0.0 (0.0) c	(9.68)	(4.85)	(112.4)
Disease index (0-4)*	0.48 (3.46) d	2.11 (8.23) ab	0.95 (5.57) c	1.98 (8.07) b	2.54 (9.15) a	0.03 (0.33) e	0.0 (0.0) e	(0.995)	(0.498)	(22.4)
Root infection index (0-4)	0.00 (0.00) c	0.10 (0.57) c	0.10 (0.57) c	1.70 (7.41) b	0.30 (1.72) c	3. 90 (11.38) a	0.0 (0.0) c	(1.345)	(0.673)	(48.6)

Arcsine transformed values for non-normal data given in parentheses. Numbers within the same row which share same the letters are not significantly different at P<0.05.*Index of Carling and Leiner (1990).

Percentage stolon infection was highest in pots inoculated with AG3PT but this was not statistically (P<0.05) different to pots inoculated with AG5. Percentage stolon infection by AG3PT and AG5 was significantly greater than that caused by AG2-1 'n'. Other AGs did not cause a statistically significant

increase in percentage stolon infection compared with the un-inoculated pots. In pots inoculated with AG3PT, AG5 or AG2-1 'n', more than 10% of stolons showed pruning, and approximately 20% more stolon pruning was caused by AG3 than by AG5 and AG2-1 'n'.

Disease indices were highest in the AG5 inoculated pots, probably due to the consistent infection of main stems by this AG. AG2-1 'n' also gave a high disease index, which reflects the fact that this isolate caused severe infections, but inconsistently. The AG3 isolate also produced a high index value (1.98). This was significantly lower ($P<0.05$) than the AG5 value (2.54) but not the AG2-1 'n' value (2.11). The AG2-1 's' isolate, X81, had a disease index value approaching one. This reflects the fact that X81 produced consistent infection but all the lesions were no larger than 5 mm (Figure 4.3). Lesion appearance also differed between AG5 and the other AGs. AG3PT and AG2-1 'n' lesions had a stripy appearance (Figure 4.4) whilst AG5 (Figure 4.5) did not.

Figure 4.3 AG2-1 's' (X81) infection of stem, arrows pointing to lesions

Figure 4.4 Segment of potato stem totally infected with AG3PT
(stem width is approximately 5 mm)

Figure 4.5 Potato stems with lesions characteristic of AG5 infection

AG8 caused the most severe root infections (index = 3.90), significantly higher than AG3 (1.7). Both AG3PT and AG8 had significantly more ($P<0.05$) root infection compared to the other AGs. Some root infections were present in isolates of AG2-1 and AG5 but these were not significantly greater than the control plots ($P<0.05$). No root, stem or stolon disease was present in the un-inoculated pots. Isolations were taken from each pot for AG confirmation. AG testing revealed no contamination from other AGs. No white-collar symptoms were observed on any plants in the glasshouse experiment.

4.3.2 Field experiment 2001

4.3.2.1 Agronomy of the growing crop

Figure 4.6 shows the percentage of emerged plants for each treatment between 18 and 24 days after planting. Plots inoculated with the AG3 (2001) isolate displayed the lowest rate of emergence, followed by those inoculated with AG8 and AG3 (1998). At 23 days after planting, plants inoculated with the AG3 (1998) isolate and the AG8 isolate showed an increased emergence rate, however, AG3 (2001) inoculated plants showed no such increase in emergence rate between 23 to 25 days. Plants inoculated with other isolates and the non-inoculated control all had similar rates of emergence.

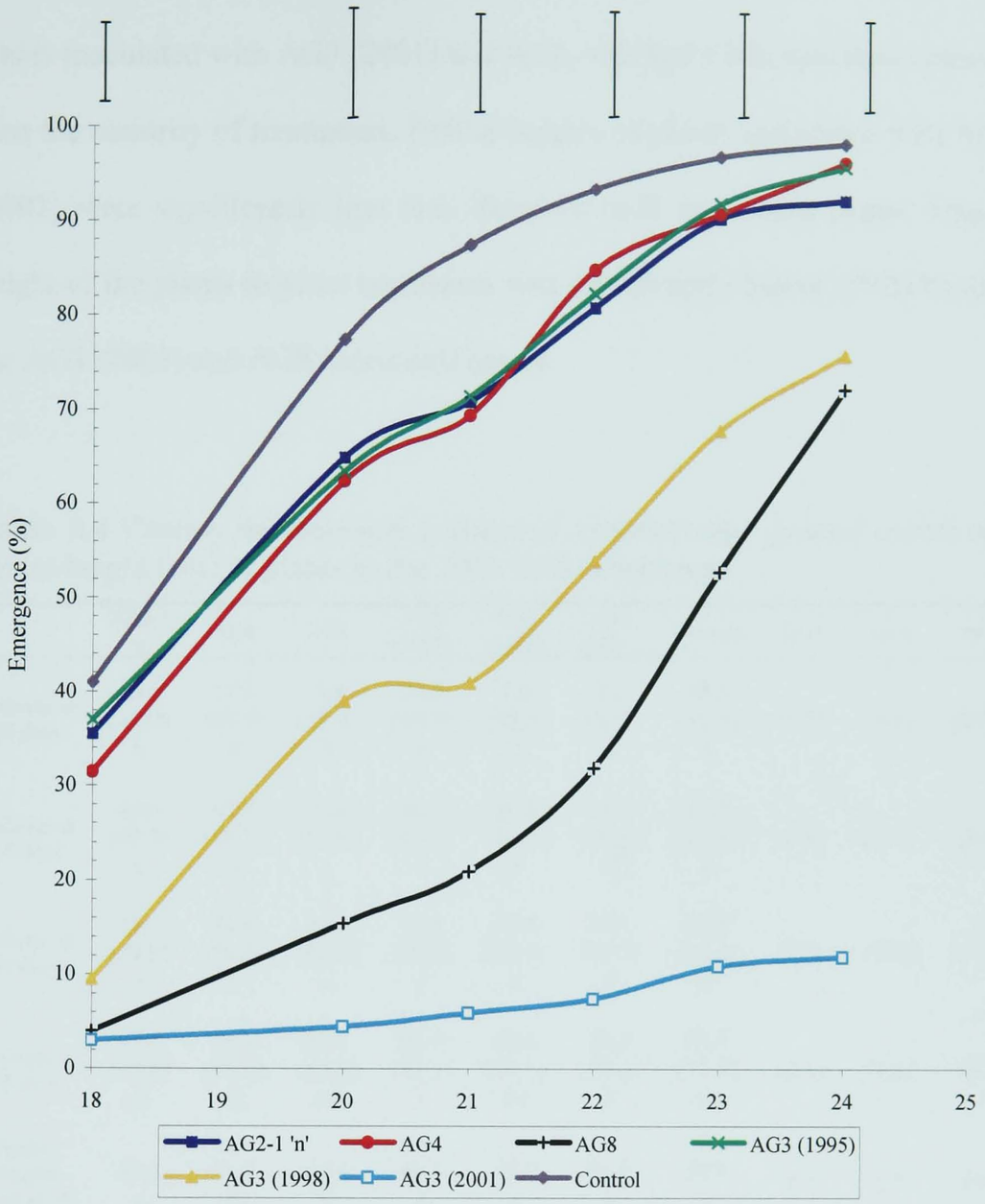


Figure 4.6 Emergence rates for each treatment in the 2001 field experiment (bars above graph representing the l.s.d between treatments for each day)

Canopy development was measured as percentage ground cover between 28 and 48 days after planting (Table 4.4). AG3 (2001) inoculated plants had significantly ($P<0.05$) less canopy development than all the other treatments between 28 to 41 days. AG8 and AG3 (1998) inoculated plants had more developed canopies than those inoculated with AG3 (2001) but still had significantly ($P<0.05$) less than that of the other treatments between 28 and 41

days. Signs of recovery were starting to show at 48 days, but at this time, plants inoculated with AG3 (2001) and AG8, still had a less developed canopy than the majority of treatments. Haulm heights of plants inoculated with AG3 (2001) were significantly less than those of AG8 inoculated plants. Haulm height of the plants in other treatments was significantly higher ($P<0.05$) than the AG3 (2001) and AG8 inoculated plants.

Table 4.4 Canopy development (measured as percentage ground cover) and haulm height (cm) of plants in the 2001 field experiment

	AG2-1 'n'	AG4	AG8	AG3 (1995)	AG3 (1998)	AG3 (2001)	Control	l.s.d	s.e.d	%cv
% Cover at 28 days	16.6 (23.9) a	17.4 (24.5) a	3.8 (10.8) b	21.2 (26.7) a	7.6 (15.5) b	1.0 (3.5) c	15.8 (23.3) a	(5.7)	(2.8)	(23.9)
% Cover at 35 days	45.6 (42.5) a	45.0 (42.1) a	20.0 (26.6) c	48.4 (44.1) a	31.8 (34.3) b	11.4 (19.5) d	43.8 (41.4) a	(3.9)	(1.9)	(8.4)
% Cover at 41 days	73.2 (58.9) a	74.6 (59.9) a	50.0 (45.0) c	74.8 (59.9) a	65.8 (54.3) b	30.4 (33.5) d	75.8 (60.6) a	(3.8)	(1.8)	(5.5)
% Cover at 48 days	93.8 (77.4) ab	96.4 (79.2) ab	82.4 (65.5) cd	96.0 (81.1) a	88.6 (71.1) bc	72.0 (58.1) d	93.4 (77.1) ab	(8.4)	(4.1)	(8.8)
Haulm Height (cm) at 48 days	49.3 a	51.7 a	39.6 b	49.7 a	47.7 a	29.5 c	50.3 a	7.7	3.7	13.0

Arcsine transformed values for non-normal data given in parentheses. Numbers within the same row which share same the letters are not significantly different at $P<0.05$.

4.3.2.2 Assessment of disease in the growing crop

Table 4.5 shows stem and root disease indices and percentage infection for stems and stolons in the 2001 field experiments. AG3 (2001) inoculated plants displayed more infection (96.5%) and pruning (82.1%) than any other treatment. Plants inoculated with AG3 (1998) exhibited statistically less

infection and pruning than those of the more recently isolated AG3 (2001), but still had significantly more infection and pruning than the other treatments. AG8, AG3 (2001) and AG3 (1998) all caused similar levels of root infection, which were significantly greater than all other treatments. In some cases (main stem infection, secondary stem infection, disease index and root infection), the AG4 and un-inoculated plants showed less signs of infection than the other treatments. All isolations of the fungus from the un-inoculated and AG4 plots were AG3, both AG2-1'n' and AG3PT isolates were recovered from the AG2-1 'n' inoculated plots and AG3 and AG8 were isolated from the plots inoculated with AG8, no AG8 was isolated from potato stems and stolons in the AG8 inoculated plots. These isolations indicate that a population of AG3PT was present in the soil prior to inoculation. No white-collar symptoms were observed on any plants in the 2001 field experiment.

Table 4.5 Main stem, secondary stem, total stem and stolon percentage infection and pruning, stem and root disease indices in the 2001 field experiment

	AG2-1 'n'	AG4	AG8	AG3 (1995)	AG3 (1998)	AG3 (2001)	Control	l.s.d	s.e.d	%cv
% Main stems infected	39.6 (38.4) cd	23.5 (28.4) d	36.9 (37.1) cd	59.2 (50.7) bc	77.8 (65.2) ab	96.5 (81.8) a	21.1 (23.9) d	(16.7)	(8.1)	(27.5)
% Main stems pruned	6.3 (11.2) c	0 (0) d	0.9 (2.4) d	1.8 (4.9) cd	43.8 (41.3) b	82.1 (65.6) a	0 (0) d	(8.0)	(3.9)	(34.1)
% Secondary stems infected	45.4 (41.9) b	12.9 (15.8) c	47.2 (40.2) b	43.0 (41.0) b	49.4 (44.7) b	80.1 (63.9) a	9.3 (15.6) c	(17.0)	(8.2)	(34.6)
% Secondary stems pruned	10.3 (13.7) c	0 (0) d	0 (0) d	0 (0) d	17.1 (23.7) b	52.4 (46.3) a	0 (0) d	(9.1)	(4.4)	(58.1)
% Total infection	42.5 (40.3) b	18.7 (25.1) c	40.7 (39.4) b	53.2 (47.1) b	59.2 (51.4) b	83.8 (66.5) a	15.9 (20.6) c	(13.3)	(6.5)	(24.6)
% Total pruning	8.5 (14.5) c	0 (0) d	0.57 (1.95) d	1.1 (3.8) d	28.2 (31.8) b	59.0 (50.2) a	0 (0) d	(7.1)	(3.4)	(37.1)
% Stolons infected	15.2 (21.7) cd	9.9 (17.9) de	24.3 (29.2) bc	26.0 (30.5) b	26.2 (30.6) b	46.0 (42.7) a	5.0 (11.3) e	(7.9)	(3.9)	(23.2)
Stem canker Key value (0-9)*	1.8 (7.6) c	1.0 (5.5) d	1.8 (7.6) c	1.8 (7.6) c	4.7 (12.5) b	7.2 (15.5) a	0.5 (4.1) e	(1.2)	(0.6)	(10.8)
Root infection index	19.5 b	8.5 c	44.0 a	7.5 c	36.5 a	48.5 a	3.5 c	10.5	5.1	33.4

Arcsine transformed values for non-normal data given in parentheses. Numbers within the same row which share same the letters are not significantly different at $P<0.05$. *Index from Back (2003).

4.3.2.3 Assessment of yields and black scurf

Table 4.6 shows the average weight and number of tubers harvested from each treatment at each grade. Uninoculated (control) plants produced the most tubers overall and significantly more than the AG8, and the AG3 (1998 and 2001) isolates. AG8 inoculated plants produced significantly less ($P<0.05$)

tubers than all the other treatments, however inoculation with AG3 (2001) resulted in a significantly lower yield by weight compared to all other treatments.

Table 4.6 Weight (kg) and number of tubers at each grading for each treatment in the 2001 field experiment

	AG2-1 'n'	AG4	AG8	AG3 (1995)	AG3 (1998)	AG3 (2001)	Control	l.s.d	s.e.d	%cv
Total weight of all tubers (kg)	12.6 ab	13.2 ab	10.5 c	13.4 a	12.1 b	9.2 d	13.8 a	1.3	0.63	8.3
Total number of tubers	118.2 ab	119.6 a	83.8 c	116.6 ab	114.0 b	101.4 b	133.0 a	17.1	8.3	11.6
Weight of tubers under 45mm (kg)	0.6 de	0.7 cd	0.4 e	0.5 de	0.8 bc	1.1 a	0.9 ab	0.19	0.09	20.2
Number of tubers under 45mm	31.0 ab	30.2 ab	17.0 b	23.0 b	35.4 a	39.4 a	35.8 a	14.1	6.8	35.6
Weight of tubers between 45-65mm (kg)	8.5 abc	7.7 abc	6.8 bc	6.9 bc	8.2 ab	6.3 c	9.1 a	1.84	0.89	18.5
Number of tubers between 45-65mm	70.4 ab	69.8 abc	55.0 c	79.4 a	68.8 a	56.4 bc	82.6 a	14.1	6.8	15.6
Weight of tubers between 65-85mm (kg)	3.8 ab	4.5 a	3.0 bd	3.3 bc	2.5 cd	1.5 d	3.4 bc	1.53	0.74	37.4
Number of tubers between 65-85mm	16.8 ab	19.6 a	11.8 bc	14.2 bc	9.8 cd	5.6 d	14.6 bc	6.2	3.0	35.9

Numbers within the same row which share the same letters are not significantly different at $P<0.05$.

The incidence and severity of black scurf was significantly higher on tubers harvested from plots inoculated with AG (2001) and AG3 (1998) at all grades (Table 4.7) when compared other treatments. Black scurf was present in all the other treatments including the uninoculated plots. All isolations of *R. solani* from black scurf symptoms on tubers obtained from any plot in the

field experiment belonged to AG3PT. Therefore it is likely that the AG3PT population present prior to planting caused the the black scurf in those plots.

Table 4.7 Average black scurf (BS) percentage incidence and average BS disease severity score for each treatment at each grading in the 2001 field experiment

	AG2-1 'n'	AG4	AG8	AG3 (1995)	AG3 (1998)	AG3 (2001)	Control	l.s.d	s.e.d	%cv
% BS incidence	9.6 (12.9) b	18.7 (20.3) b	10.4 (15.3) b	5.7 (8.3) b	97.3 (81.9) a	94.7 (78.8) a	17.8 (21.9) b	(17.19)	(8.39)	(38.8)
BS disease score (all tubers)	0.21 (1.79) b	0.35 (2.61) b	0.22 (2.20) b	0.10 (1.04) b	2.01 (8.14) a	1.93 (7.98) a	0.32 (2.80) b	(2.17)	(1.06)	(44.1)
% BS incidence (under 45mm)	10.7 (12.4) b	11.7 (15.2) b	10.6 (13.9) b	3.3 (6.5) b	91.1 (75.1) a	90.4 (74.8) a	7.0 (13.2) b	(16.85)	(8.23)	(43.1)
BS disease score (under 45mm)	0.24 (1.74) b	0.24 (2.11) b	0.21 (1.97) b	0.05 (0.81) b	1.85 (7.80) a	1.80 (7.68) a	0.14 (1.82) b	(2.06)	(1.005)	(46.5)
% BS incidence (45 to 65mm)	8.6 (12.0) b	21.1 (21.8) b	9.9 (13.6) b	6.4 (8.7) b	99.7 (88.7) a	96.8 (82.4) a	22.0 (24.7) b	(18.13)	(8.85)	(38.9)
BS disease score (45 to 65mm)	0.19 (1.69) b	0.39 (2.75) b	0.22 (1.93) b	0.11 (1.06) b	2.05 (8.22) a	1.98 (8.08) a	0.38 (3.07) b	(2.29)	(1.117)	(46.1)
% BS incidence (65-85mm)	12.7 (15.7) b	20.6 (21.4) b	12.8 (16.4) b	6.9 (9.3) b	100.0 (90.0) a	80.0 (72.0) a	12.9 (16.5) b	(26.67)	(13.02)	(59.7)
BS disease score (65-85mm)	0.26 (2.06) b	0.40 (2.78) b	0.26 (2.24) b	0.13 (1.22) b	2.226 (8.56) a	2.05 (7.33) a	0.26 (2.23) b	(3.19)	(1.559)	(65.3)

Arcsine transformed values for non-normal data given in parentheses. Numbers within the same row which share the same letters are not significantly different at $P<0.05$.

4.3.3 Field experiment 2003

4.3.3.1 Agronomy of the growing crop

The percentage of emerged plants for each treatment between 17 and 26 days after planting is shown in Figure 4.7. Plants inoculated with AG8 showed the lowest rate of emergence between 17 and 25 days. AG3PT inoculated plants showed a similar percentage emergence to the AG8 inoculated plants up until 20 days. From 20 days after planting the percentage of emerged plants was greatest in the uninoculated plots.

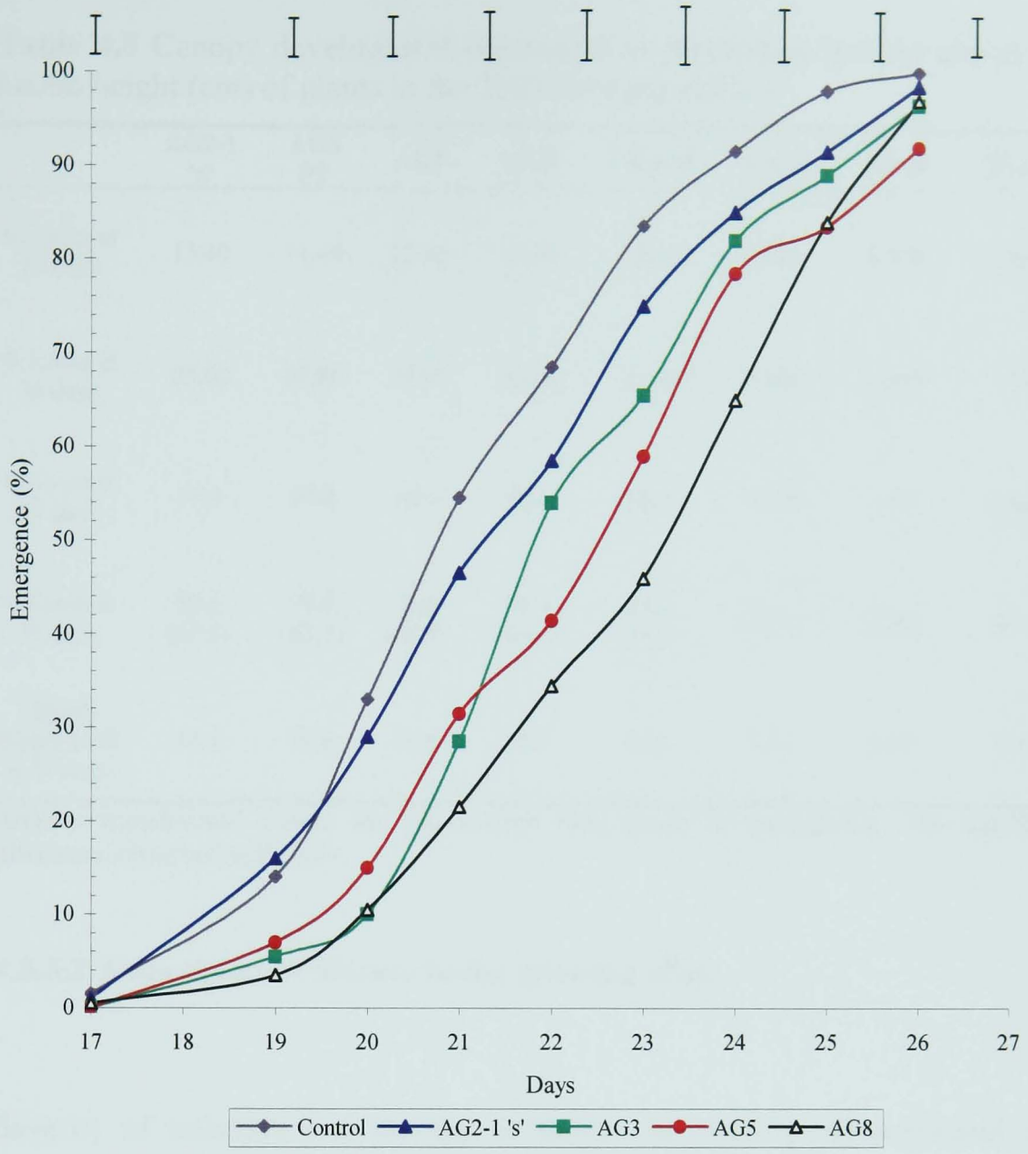


Figure 4.7 Emergence for each treatment in the 2003 field experiment (bars above graph representing the l.s.d between treatments for each day)

No significant differences in canopy development (measured as percentage ground cover) or haulm height were observed between treatments (Table 4.8), however height and canopy development was generally greater in the un-inoculated plots.

Table 4.8 Canopy development (measured as percentage ground cover) and haulm height (cm) of plants in the 2003 field experiment

	AG2-1 's'	AG3 PT	AG5	AG8	Control	l.s.d	s.e.d	%cv
% Cover at 29 days	15.40	11.40	12.40	11.00	12.40	4.943	2.370	29.9
% Cover at 35 days	25.00	22.80	25.00	22.20	27.20	5.600	2.685	17.4
% Cover at 45 days	57.4	47.8	56.4	48.6	56.4	10.39	4.98	14.8
% Cover at 51 days	84.6 (67.5)	79.0 (63.1)	78.8 (62.9)	74.4 (60.0)	85.0 (67.5)	(7.94)	(3.80)	(9.4)
Haulm Height (cm) at 51 days	61.1	56.9	56.1	55.2	62.5	7.04	3.37	9.1

Arcsine transformed values for non-normal data given in parentheses. No significant difference observed at $P<0.05$.

4.3.3.2 Assessment of disease in the growing crop

Severity of infection and pruning of stems and stolons (all expressed as a percentage of the total number of plants assessed) and average disease indices for all treatments are shown in Table 4.9. No infection was observed on plants in the control (un-inoculated) plots. Over 80% of the main stems in the AG3 and AG5 inoculated plots were infected, this severity of infection was significantly greater ($P<0.05$) than all other treatments. Main stem pruning and secondary stem infection and pruning was only observed in the AG3 inoculated plots.

AG5 caused a significantly higher percentage of stolon infections than AG3PT, and both AG5 (55.9%) and AG3PT (21.9%) caused more stolon infection than the other treatments. AG3PT caused significantly more stolon

pruning than AG5. No stolon pruning was present in any of the other treatments.

Table 4.9 Main stem, secondary stem, total stem and stolon percentage infection and pruning, stem and root disease indices in the 2003 field experiment

	AG2-1 's'	AG3 PT	AG5	AG8	Control	l.s.d	s.e.d	%cv
% Main stems infected	14.8 (20.3) b	83.4 (66.7) a	90.8 (73.6) a	5.8 (10.3) bc	0.0 (0.0) c	(11.26)	(5.4)	(25.0)
% Main stems pruned	0.0 (0.0) b	28.65 (32.3) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	(2.330)	(1.117)	(27.4)
% Secondary stems infected	0.0 (0.0) b	38.0 (34.9) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	(13.77)	(6.6)	(149.5)
% Secondary stems pruned	0.0 (0.0) b	8.9 (11.1) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	(9.24)	(4.43)	(316.1)
% Total infection	14.8 (20.3) c	71.0 (57.6) b	90.8 (73.6) a	5.8 (10.3) cd	0.0 (0.0) d	(10.95)	(5.25)	(25.7)
% Total pruning	0.0 (0.0) b	23.14 (28.7) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b	(0.6511)	(0.3122)	(8.6)
% Stolons infected	0.1 (0.8) c	21.9 (26.9) b	55.9 (48.5) a	0.1 (1.0) c	0.0 (0.0) c	(8.59)	(4.12)	(42.2)
% Stolons pruned	0.00 (0.0) c	8.21 (16.45) a	0.77 (3.88) b	0.00 (0.00) c	0.00 (0.00) c	(2.79)	(1.338)	(52.0)
Stem disease index*	0.47 (3.46) b	1.98 (8.07) a	1.90 (7.92) a	0.05 (0.95) c	0.00 (0.00) c	(1.43)	(0.684)	(26.5)
Root infection index	0.04 (0.51) c	1.18 (6.19) b	0.20 (1.54) c	2.26 (8.63) a	0.00 (0.00) c	(1.63)	(0.782)	(36.6)

Arcsine transformed values for non-normal data given in parenthesis. Numbers within the same row which share the same letters are not significantly different at $P<0.05$. *Index of Carling and Leiner (1990).

Using the stem disease index of Carling and Leiner (1990), AG3 and AG5 had similar scores, both approaching the value of two. This was probably due to consistent infection by the AG5 isolate compared to less frequent but severe

infections and also sometimes stem pruning by AG3. The AG2-1 's' isolate had a disease index value approaching 0.5, reflecting the abundance of the small lesions no bigger than 5 mm.

The average root disease index of AG8 inoculated plants (2.26), was significantly greater ($P<0.05$) than that in all other treatments, including that of AG3PT inoculated plants (1.18). Small but insignificant levels of root infection were observed with the AG2-1 's' and AG5 treatments. Isolations confirmed no other AGs except those used for inoculation were present in each plot.

The white-collar symptom was only observed in plants infected with AG3PT, white-collar was not observed in plants from other treatments.

4.3.3.3 Assessment of yields and black scurf

Weight and number of tubers is shown in Table 4.10 and shows that the un-inoculated plots produced more tubers by number and weight than the other treatments. AG8 infected plants produced significantly less ($P<0.05$) tubers compared to all other treatments except AG3.

Table 4.10 Weight (kg) and number of tubers harvested at each grade for each treatment in the 2003 field experiment

	AG2-1 's'	AG3 PT	AG5	AG8	Control	l.s.d	s.e.d	%cv
Total weight of all tubers (kg)	13.73 b	13.21 b	13.40 b	12.19 b	15.65 a	1.639	0.786	9.1
Number of all tubers	121.6 ab	109.2 bc	115.8 b	93.0 c	137.4 a	18.88	9.05	12.4
Weight of tubers under 45mm (kg)	0.922 a	0.598 b	0.809 ab	0.528 b	0.952 a	0.2895	0.1388	28.8
Number of tubers under 45mm	25.8 a	18.6 ab	24.0 a	15.6 b	25.8 a	8.14	3.90	28.1
Weight of tubers between 45-65mm (kg)	9.18 ab	8.28 bc	8.56 bc	7.05 c	10.86 a	1.843	0.883	15.9
Number of tubers between 45-65mm	80.4 ab	70.4 bc	74.8 bc	59.2 c	93.8 a	17.29	8.29	17.3
Weight of tubers between 65-85mm (kg)	3.63 a	4.33 a	4.02 a	4.61 a	3.84 a	1.511	0.725	28.0
Number of tubers between 65-85mm	15.4 a	20.2 a	17.0 a	18.2 a	17.8 a	7.51	3.60	32.1

Arcsine transformed values for non-normal data given in parenthesis. Numbers within the same row which share the same letters are not significantly different at $P<0.05$.

The incidence and disease score of black scurf was highest in the AG3 inoculated plots (83% incidence and score of 1.5), this was significantly greater than the other treatments (Table 4.11). Other treatments did not cause significant levels of black scurf, and all cultures isolated from black scurf present in the AG8 and un-inoculated plots were identified as AG3PT. This indicates that AG3PT was already present in some small proportions in the soil, on the seed tubers or cross-contamination occurred from the AG3PT plots. AG2-1 's' and AG5 were both isolated from progeny tubers of plants

inoculated with those AGs indicating that these groups have an ability to cause some black scurf.

Table 4.11 Black scurf incidence and score at each grading in the 2003 field experiment

	AG2-1 's'	AG3 PT	AG5	AG8	Control	l.s.d	s.e.d	%cv
All tubers Incidence	0.9[1] (3.2) b	83.9[2] (67.6) a	3.2[3] (9.8) b	3.6[2] (6.0) b	0.3[2] (1.4) b	(9.25)	(4.43)	(39.8)
All tubers Score	0.012 (0.39) b	1.547 (7.11) a	0.049 (1.22) b	0.076 (0.85) b	0.005 (0.18) b	(1.11)	(0.53)	(43.0)
Under 45mm incidence	0.9 (2.4) b	75.5 (61.8) a	6.2 (9.2) b	3.5 (5.0) b	0.0 (0.0) b	(12.86)	(6.17)	(62.2)
Under 45mm Score	0.009 (0.24) b	1.300 (6.46) a	0.089 (1.07) b	0.094 (0.79) b	0.0 (0.0) b	(1.572)	(0.754)	(69.6)
Between 45-65mm incidence	0.8 (3.2) b	84.8 (68.2) a	1.6 (6.3) b	4.2 (6.7) b	0.5 (1.9) b	(9.50)	(4.56)	(41.8)
Between 45-65mm score	0.013 (0.42) b	1.570 (7.16) a	0.025 (0.80) b	0.081 (0.90) b	0.008 (0.23) b	(1.140)	(0.547)	(45.4)
Between 65-85mm incidence	1.3 (3.0) b	86.3 (71.2) a	3.0 (4.6) b	1.3 (3.0) b	0.0 (0.0) b	(11.32)	(5.43)	(52.5)
Between 65-85mm score	0.013 (0.30) b	1.625 (7.30) a	0.050 (0.57) b	0.040 (0.51) b	0.000 (0.00) b	(1.149)	(0.551)	(50.1)

Numbers within the same row which share the same letters are not significantly different at $P<0.05$.

[1]All isolates taken were AG2-1's'; [2]All isolates taken were AG3PT; [3]All isolates taken were AG5 except one isolate belonging to AG2-1's'

4.4 Discussion

The field and glasshouse experiments conducted in this study revealed that there are considerable differences in both the type and severity of potato disease caused by individual AGs. For example, isolates of AG2-1, AG3PT and AG5 are all capable of causing severe infections of stems, whilst AG8 almost exclusively infects roots. AG3PT also appears to be the only AG that can cause significant amounts of black scurf, other AGs tested produced little or no black scurf in the field experiments.

This is the first study comparing potato disease initiated by different AGs in the field, however similarities do exist between this and previous studies under controlled conditions. For example AG3, was highly virulent to potatoes as in previous studies (Carling and Leiner, 1986; Chand and Logan, 1983; Carling and Leiner, 1990; Petkowski and de Boer, 2001; Bains and Bisht, 1995; Balali *et al.,* 1995). Also the AG5 isolate, exhibited severe infection, which was concordant with previous studies (Carling and Leiner, 1990; Bains and Bisht, 1995; Balai *et al.,* 1995). In both field and glasshouse experiments, potato infection by AG8 was almost exclusively limited to roots, which was also previously observed (Carling and Leiner, 1990; Hide and Fimager, 1990).

A noticeable difference is that AG4 did not cause severe stem infections in this study as was observed previously in published studies (Anguiz and Martin, 1989; Bains and Bisht, 1995; Balali *et al.,* 1995). There are several

reasons for this lack of virulence, including that AG4 usually causes severe infection in warmer climates (Anguiz and Martin, 1989). Also AG4 is a heterogeneous group associated with many hosts (Sneh *et al.*, 1991) and the strain of the isolate used in the 2001 field experiment may not be particularly virulent towards potato. However, the likely reason for observing little infection is that the isolate had been in continuous subculture for some time and virulence may have been lost.

The importance of using recent isolates is exhibited in the 2001 field experiment, where three different AG3PT isolates were used. The most recent isolate caused significantly more severe disease than the other two, with the oldest isolates causing least severe disease. This shows the importance of using recent or freshly passaged isolates. In this study, the choice of isolates was different between experiments; isolates used in the 2001 field experiment were chosen solely on the basis of availability. In the second field experiment and the glasshouse experiment, isolates collected from the investigation in Chapter 3 could be used, as these were most relevant to test and isolates were recently passaged through the host. No subsequent experiments were performed with the AG4 isolate, as passaging through a potato plant was not successful. Where numerous isolates were available e.g. for AG2-1 'n' and 's' groups and also AG3PT, interpretation of data from the pathogenicity test in Chapter 3 enabled the choice of isolates that were representative of those groups.

The pathogenicity tests of Chapter 3 and the 2001 field experiment did show that variation can occur between isolates belonging to the same AG. It may be suggested that the difference observed here between the AGs could be attributed to inter-isolate variation within an AG as opposed to inter-AG variation. However various steps had been taken to limit this, for example the choice of representative isolates on the basis of the pathogenicity test data in Chapter 3 and also passaging of isolates.

One approach to limit the effect on inter-isolate variation may possibly be to inoculate each plant with several isolates of the same AG. However, this method may instigate other problems, for example the interaction of different isolates within the same AG may affect virulence, killing (C2) reactions may occur between the isolates, the sexual stage of the life cycle may be initiated or an avirulent isolate may cause the plant to develop systemic acquired resistance. Also, in most situations only one isolate is interacting with the potato plant, therefore using one isolate is perhaps best representative of a field situation.

The choice of representative isolates is perhaps the best approach after increasing the size of the experiment to test multiple isolates within an AG, the size of which would be prohibitively expensive. This study has also indicated differences in the type of disease these AGs can produce, such as AG8 causing root disease and not stem canker or black scurf like AG3PT. The ability to cause this type of disease as opposed to disease severity is likely to be indicative of all members of that AG.

One AG where variation did occur was AG2-1. The previous chapter indicated this was a diverse group with the presence of three lengths of the IGS1 region. In this study the isolate of AG2-1 's' caused 'little lesions' i.e. narrow lesions no longer than 5 mm in length. AG2-1 'n', had the ability to cause significant stem infections, where pruning of the main stem is not uncommon. The isolate of AG2-1 'l' also displayed the ability to initiate stem pruning in the glasshouse experiment but this was uncommon. These differences may explain why in the studies of Carling and Leiner (1986; 1990) and Chand and Logan (1983) infection of stems by AG2-1 was not severe, whereas Petkowski and de Boer (2001) observed severe stem infection with AG2-1 isolates. It may have been possible that Petkowski and de Boer (2001) used isolates belonging to the AG2-1 'n' type. The discovery of isolates which caused the 'little lesions' in this study led to the use of the disease index of Carling and Leiner (1990) instead of Back (2003). The key of Carling and Leiner was more suited to score for the frequently occurring small lesions.

In all experiments in this study the symptom of the sexual stage: 'white-collar' was not observed amongst AG2-1 infected plants, of all IGS1 types. In a previous study by Carling and Leiner (1986), the white-collar symptom was uncommon with AG2-1 stem infection. Where white-collar was present on AG2-1 infected stems, few basidia were present (Carling and Leiner, 1986). Because of this observation Carling and Leiner (1986) suggested that potato was not a primary host for AG2-1. These results suggest that due to the differences in disease severity between the AG2-1 types, there may be two or

three primary hosts for AG2-1, correlating with the different IGS1 types. Further work needs to be done in this area as despite the lack of sclerotia, certain types of AG2-1 can instigate severe stem infection which may be causing yield losses of at least 10%. Determination of the alternative host may help construct an efficient crop rotation strategy to negate such losses.

The other AGs tested in this study; despite being able to cause significant infection of stems or roots, did not cause severe black scurf, unlike AG3PT. This was also observed in previous glasshouse studies (Campion *et al.,* 2003; Stack *et al.,* 1999; Balali *et al.,* 1995). These other AGs do have the ability to create sclerotia as they were often observed when grown *in vitro* on PDA during this study. This suggests that AG3PT has evolved some specificity to potato, as unlike the other AGs tested, it has the ability to form a considerable number of sclerotia on potato tubers. Transmission through infected seed is an efficient mechanism of long distance dispersal, as observed by Ceresini *et al.* (2003). The other AGs tested here may be limited to dispersal through infected soil and/or stem and stolon tissue.

Previously, the importance of stem and stolon infection has been emphasised in the reduction of yield (Gudmested *et al.,* 1989). In this study, plants infected with AG8 where root infection predominated displayed considerable yield losses up to 24% by weight. In contrast, despite severe stem and stolon infections, yield losses with AG5 were only 14%, even stem infection resulting in small lesions with the AG2-1 's' isolate resulted in a yield loss of 12% (compared to the uninoculated control). Yield losses were also severe

with AG3PT, up to 33% in 2001, however in 2003 a smaller yield loss was observed (15%). The ability of AG3PT to cause severe yield losses as compared to AG2-1 and AG5 may be explained by the ability of this AG to cause moderate root infections, unlike AG2-1 and AG5. Carling and Leiner (1990) also observed that AG3 could cause moderate root infections whilst AG5 did not. This suggests that root infection has a major role in causing yield losses. In this study, the importance of root infection was evident soon after planting, as emergence rates for AG3PT and AG8 inoculated plants were considerably lower than other treatments in both field experiments. Due to the difficulty of working with roots, this aspect of the disease may have been overlooked and further work is required to study the significance of root infections.

Infection by AG3PT, in addition to causing quantitative damage through yield loss, also causes qualitative damage with black scurf, resulting in additional economic loss. However in previous studies, qualitative damage was also observed with tuber deformation associated with AG2-1 and AG5 infections (Campion *et al.,* 2003). No deformation of tubers was observed with the AGs tested in this study, this may be attributed to varietal differences. Campion *et al.* (2003) found differences in severity and incidence of tuber deformations between varieties. The variety used in this study, Désirée, may therefore possess some degree of insensitivity to these deformations.

AG5 differed from AG2-1 'n' and AG3PT infection in both lesion appearance and frequency of stem pruning. In the glasshouse and field experiments

pruning of up to 52% and 20% was observed for AG3PT and AG2-1 'n' isolates respectively, but despite consistent and severe stem infections, main stem pruning in AG5 inoculated plants was never greater than 3.3% and no pruning was observed in the 2003 field experiment for AG5. Percentage pruning was also low in the pathogenicity tests in Chapter 3. It could possibly be that the appearance of the lesion and the ability to prune are linked, perhaps AG5 does not posses a particular enzyme which can actually prune the plant. Virulence in AG3 was previously observed to correlate with the ability to produce phenyl-acetic acid derivatives *in vitro* (Tavantzis *et al.,* 1989). It would be interesting to quantify which type of phenyl-acetic acid derivatives AG5 isolates produce and compare this with other AGs. Differences in the amount and type of these compounds may explain the difference in pruning ability.

This study has shown the usefulness of field and glasshouse to compare disease initiated with different AGs. Field experiments allow the disease to be studied in conditions close to how infection occurs naturally. Glasshouse experiments are less like the natural conditions but offer the advantage of greater control over the environment. A major limitation of the 2001 field experiment was that a substantial amount of background contamination of AG3 was present; such contamination was not present in the glasshouse experiment. Despite the presence of contamination, the 2001 field experiment did show differences between the type and severity of disease caused by the different AGs. It also showed that AG3PT out competed other AGs present during the infection of potato; this was observed previously by Stack *et al.*

(1999) who inoculated potato with both AG3 and AG5, and found that AG3 predominated.

It is interesting to note that both fields used in the field experiments had not grown potatoes since 1985. The previous crop was sugar beet in the field used for the 2001 field experiment, whilst wheat was the previous crop in the field used for the 2003 field experiment. AG3 was associated with sugar beet by Windels *et al.* (1997), who observed that 85% of sugar beet isolates were AG3. These observations suggest that sugar beet can transmit propagules of AG3 to the soil; alternatively planting of the crop may increase the amount of AG3 already present in the field. However, other studies investigating AGs present in sugar beet indicated that AG4 was the predominant group (Rush *et al.,* 1994; Windels *et al.,* 1989). This work was conducted in North America and its relevance to the UK may be limited, therefore further work is needed to determine if sugar beet in the UK is associated with rhizoctonia potato disease.

In conclusion, this study has shown that all AGs of *R. solani* tested here have some adverse affect on potato yields. AG3PT has the ability to cause both quantitative and qualitative damage to the crop and may perhaps be considered most important. This work has also indicated that AG8 could be an important consideration due to the ability to severely infect roots and cause yield losses of over 20%. This study shows that infection of roots may be a key factor in yield losses whereas previously stem and stolon cankers were thought to be of major importance.

5. Phylogenetic relationships of *Rhizoctonia solani* anastomosis groups associated with potato based on ribosomal DNA sequences.

5.1 Introduction

5.1.1 Techniques used in phylogenetic analysis

Phylogenetic analysis is usually associated with DNA sequences, however it predates DNA sequencing and derives from traditional methods of classifying organisms (Brown, 1999). Originally, morphological data was used to construct a classification or phylogeny, but molecular data, in the form of immunological tests was used at an early stage to deduce the relationships between varieties of animals (Nuttall, 1904). Immunological data involves measurements of the cross-reactivity observed when a protein specific antibody from one organism is mixed with the same protein from another organism. If the proteins are similar then this will result in a high degree of cross-reactivity, thus providing similarity data for analysis. Enzymes and proteins can also be analysed using electrophoresis and their electrophoresis properties compared. However studies with DNA have given the most insight into phylogenetic relationships.

Several techniques are currently available to analyse DNA. Early studies utilised data from DNA/DNA hybridisation assays. This involves denaturing DNA samples from the two organisms under comparison. The denatured samples are then mixed to form a hybrid molecule and the stability of these

molecules subsequently measured by determining the melting temperature. Similar samples produce more stable hybrids and thus having a higher melting temperature.

The ability of restriction enzymes to cleave DNA at specific sites has also been utilised, so called restriction fragment length polymorphism (RFLP). RFLP analysis involves the addition of one or more restriction enzymes to large amounts of DNA, which cleave the DNA at specific sites. Gel electrophoresis of the products reveals a fingerprint made up of DNA fragments of different sizes as different DNA sequences will vary in the position and number of restriction enzyme sites. Such analysis has been done with whole genomic DNA, or on specific regions such as rDNA. PCR is often used to specifically amplify such a region (PCR-RFLP).

DNA profiling (or 'fingerprinting') techniques such as random amplified polymorphic DNA (RAPD) analysis (Williams *et al.,* 1990; Williams and Kubleik, 1991) have been used. RAPD analysis uses short arbitrary primers in PCR that bind to complementary DNA throughout parts of the genome. This results in the amplification of variable numbers of DNA fragments, each with variable sizes. Band size and number is dependent on the genome, the primers used and the PCR conditions used. From such a profile, a similarity matrix can be constructed and DNA similarity deduced.

The reliability and reproducibility of results of RAPD analysis has been questioned, the random nature of the annealing of the short arbitrary primers

mean that the profile generated is profoundly influenced by reaction conditions such as DNA quality and the presence of contaminants. Consequently a more robust profiling method has been used which combines the RFLP technique with PCR, amplified fragment length polymorphisms or AFLP (Vos *et al.,* 1995)

AFLPs are generated by ligation of known sequence adapter molecules to a population of doubly digested DNA fragments. A specific subset of the fragments are PCR amplified using adapter homologous primers to which additional selective nucleotides are added to the 3' end (Vos *et al.,* 1995). Products are usually smaller than conventional PCR and undergo electrophoresis on a polyacrylamide gel. Radiolabelling of the primers enable banding to be visualised with an autoradiogram of the gel.

Sequence data however, both of proteins but more commonly DNA, is the main choice in phylogenetic analysis of fungi. Most DNA sequencing is now automated but often based on the original dideoxynucleotide system or chain termination method devised by Sanger *et al.* (1977). The sequence of a single stranded DNA is determined by enzymatic synthesis of complementary polynucletoide chains. Both deoxynucleotides and dideoxynucleotide are present and when a diedeoxynucletide is incorporated the chain is terminated. This results in a population of DNA chains differing by just one base pair in length, comparison of these chains (i.e. by electrophoresis) allows the position of each nucleotide to be deduced.

Several regions of DNA have been used in phylogenetic analysis including ribosomal DNA, the β-tubulin gene, hydrophobin genes and mitochondrial DNA (Burnett, 2003). Sequences of rDNA are probably the most common sequence utilised in phylogenetic studies, rDNA lends itself to such studies as it consists of both variable and conserved regions (Figure 5.1) and is often an ideal size for sequencing.

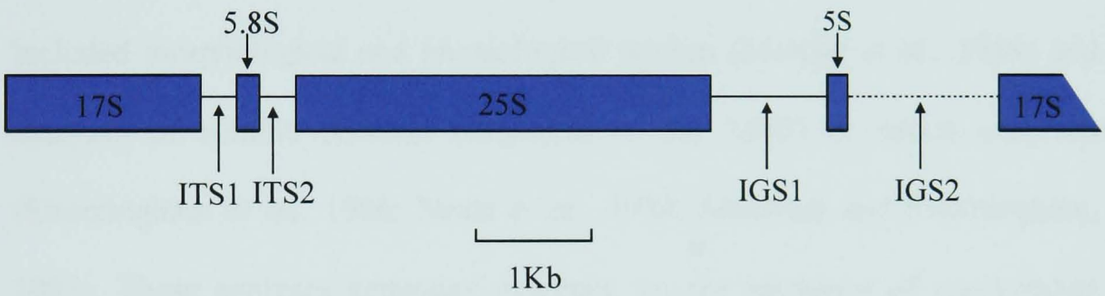


Figure 5.1 Arrangement of nuclear rDNA in basidomycete fungi.
Coding region (conserved) in blue blocks, non-coding (variable) sequences unblocked. Sizes approximate for basidiomycete fungi. Broken line indicates uncertainty in the size of IGS2 region – varies between 1-4 Kb in fungi.

In the nuclear genome of basidiomycetes, ribosomal genes are arranged in a series of repeated units that include genes for the 17S, 25S, 5.8S and 5S rRNA subunits (Vilgalys and Gonzalez, 1990). These genes evolve relatively slowly and are useful for studying distantly related organisms (White *et al.*, 1990). However non-coding 'spacer' sequences exist between genes and they are not highly conserved. The 25S to 17S rRNA subunits are separated by the intergenic spacer (IGS) region, this region contains the 5S rRNA gene, which is absent in some ascomycetes. Situated between 17S and 25S rRNA subunits is the 5.8S subunit, which is flanked by internal transcribed spacer (ITS)

sequences, ITS1 and ITS2. As both ITS and IGS regions are non-coding, the absence of selection pressures has resulted in high variability at both species and strain level, this variation is greater in the IGS region (Martin *et al.*, 1999).

5.1.2 Phylogenetic analysis in *Rhizoctonia solani*

A range of techniques have been used to study the relatedness of *R. solani*, particularly the relationships between AGs. Non-DNA based techniques have included morphological and physiological studies (Mordue *et al.*, 1989) and analyses of soluble proteins (Reynolds *et al.*, 1983) or pectic enzymes (Sweetingham *et al.*, 1986; Neate *et al.*, 1988; MacNish and Sweetingham, 1993). These analyses generated evidence for the existence of anastomosis groups and elucidated subgroups. For example, MacNish and Sweetingham (1993) assigned isolates of AG8 to one of five subgroups based on pectic zymogram patterns. Isolates of AG2 were grouped into one of five sets using isozyme analysis and RFLPs (Liu and Sinclair, 1992). Isozyme analysis was also used to differentiate between isolates of AG3 and AG9 (Laroche *et al.*, 1992) and also differentiate North American AG3 isolates from those from Britain and Japan. Cellular fatty acid analysis has also been used to differentiate between subgroups within AG2-2 (Stevens-Johnk and Jones, 1993), AG3 (Stevens-Johnk *et al.*, 1993) and AG4 (Stevens-Johnk and Jones, 2001).

DNA hybridisation assays also support AGs and subgroups. Hybridisation values between AGs are usually less than 30% whilst within an AG they are usually greater than 70%. Reduced hybridisation values are observed between subgroups, whereas isolates belonging to the same subgroup have hybridisation values of 80 to 100% (Vilgalys and Cubeta, 1994).

Early RFLP work distinguished between AGs but failed to distinguish between subgroups (O'Brien, 1994). Jabaji-Hare *et al.* (1990) used RFLPs to distinguish between AGs but failed to segregate subgroups of AG2 and AG4 although they did observe inter-group variation within AG3. Later studies also found genetic differences between AG3 isolates from different hosts using RFLPs of the ITS and IGS regions and AFLPs (of the whole genome), supporting the segregation of AG3 of potato and tobacco isolates into AG3PT and AG3TB respectively (Ceresini *et al.,* 1999; Ceresini *et al.,* 2002b). Other subsequent studies used RFLPs of the rDNA to show existence of variation within AG3 (Liu and Sinclair, 1992) and AG4 (Meinhardt *et al.,* 2002). Recently rDNA ITS RFLPs were successfully correlated to virulence, sclerotia and cultural characteristics within AG1 and AG2 isolates causing web blight of *Phaseolus vulgaris* (Godoy-Lutz *et al.,* 2003).

PCR RAPDs have also been used successfully to provide support for subgroups within *Rhizoctonia*, Duncan *et al.* (1993) and Toda *et al.* (1999) could distinguish between AGs and subgroups using RAPDs. Further classification within subgroups could be determined with RAPD profiling, Zens and Dehne (1999) and Toda *et al.* (2004) determined the existence of

groups within AG2-2. Studies have shown that virulence may or may not be correlated to RAPD groups. For example, Pascual *et al.* (2000) could use RAPD profile data of AG1-IA isolates to construct a dendrogram and noticed a clustering of virulent isolates. However Yang *et al.* (1995) could not distinguish between virulent and weakly virulent AG8 isolates using RAPD.

Extensive studies comparing the rDNA ITS sequences within *R. solani* have been performed, leading to over two hundred *R. solani* rDNA ITS sequences representative of the first twelve AGs being deposited on GenBank (as of November 2004). Comparison of ITS sequences have supported the existence of AGs, the first extensive sequence comparison was by Kuninaga *et al.* (1997) who compared sequences of AGs 1 to 9. They found considerable ITS sequence variation between the AGs and provided support for the existence of the first three AG1 subgroups, initial evidence for the existence of tobacco and potato subgroups within AG3 and the segregation of AG4 into HG-I and HG-II. In a recent phylogenetic analysis of the ITS sequences of AGs 1 to 12, as well as binucleate *Rhizoctonia* isolates, Gonzalez *et al.* (2001) observed good support for previously defined subgroups and that some evidence that isolates of *Ceratobasidium* were closely related to some AGs of *R. solani*. Roberts (1999) also suggests that the two genera are difficult to segregate on the basis of ITS sequence comparison, however the segregation is supported by differences in morphology.

Some rDNA ITS sequence comparison studies have concentrated on defining subgroups. A more detailed study by Kuninaga *et al.* (2000a) confirmed the

existence of tobacco and potato subgroups. Boysen *et al.* (1996) segregated isolates of AG4 into three groups that correlated well with habitat and virulence. Later studies confirmed this, firstly Kuramae *et al.* (2003) found that tomato and melon isolates relate to HG-I and broccoli and spinach isolates relate to HG-III using rDNA ITS sequence analysis. Secondly Hsiang and Dean (2001), used ITS sequences to assign *R. solani* isolates from *Poa annua* to AG4 HG-III. The segregation of AG4 into three groups is also supported by fatty acid analysis (Stevens-Johnk and Jones, 2001). Recently, a new subgroup within AG6 was determined on the basis of ITS sequences and DNA amplification fingerprinting, this subgroup AG6 LRC, unlike other isolates of AG6 is not pathogenic to wheat and causes lucerne root canker (Anderson *et al.,* 2004)

Extensive DNA sequence studies have been done with AG2, supporting data from RAPD studies (Zens and Dehne, 1999; Toda *et al.,* 2004). Salazaar *et al.* (2000a) found that rDNA ITS sequences supported the subgroupings of AG2-2. ITS sequences were also used to assign tobacco isolates, which previously were thought to be related to AG2 (Nicoletti *et al.,* 1999) into AG2-1 despite low hyphal fusion frequencies with other AG2-1 isolates (Kuninaga *et al.,* 2000b). Analysis of ITS sequence in conjunction with hyphal fusion supported the existence of subgroups within AG2 and the inclusion of AGBI isolates in AG2, which was subsequently known as AG2-BI (Carling *et al.,* 2002a).

Therefore comparison of ITS sequences is a powerful tool for studying the variation within and between AGs of *R. solani.* This objective of this study

was to compare ITS sequence data of potato *R. solani* isolates with *R. solani* sequence data of isolates from other hosts. This data was used to construct neighbour joining trees inferring phylogenetic relationships for isolates of AG2-1, AG3, AG4, AG5 and AG8. These particular AGs were selected for analysis due to their association with potato diseases.

5.2 Materials and methods

5.2.1 DNA sequencing

DNA extraction from pure culture and PCR was performed as described in Chapter 2. To prepare DNA for sequencing of the rDNA ITS region, PCR was performed with ITS4 and ITS5 primers (White *et al.*, 1990) with an annealing temperature of 58°C. Primer sites are displayed in Figure 5.2, which also displays the primer sites for the IGS1 region.

Figure 5.2 Location of primer sites within rDNA (primers site and direction in red arrows, not to scale)

PCR products were electrophoresed through a 1% (w/v) low melting point agarose gel (containing 0.5 μg ml^{-1} ethidium bromide) in TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8). The DNA band for sequencing was excised from the gel and the DNA purified using Wizard PCR Preps (Promega, Southampton, UK) according to the manufacturer's instructions. Purified DNA was then quantified against known standards using a gel documentation system (Gel Doc 1000, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and diluted to the correct concentration for sequencing by MWG Biotech AG (Ebersberg, Germany). Sequencing was performed in both directions and a consensus sequence was constructed using BioEdit 5.0.

5.2.2 Sequence analysis

DNA alignments were performed using ClustalW (Thompson *et al.,* 1994) and edited visually using BioEdit 5.0 (Hall, 1999). Trees showing the phylogenetic relatedness between isolates were constructed with the MEGA 2.1 software (Kumar *et al.,* 2001). Trees were constructed according to Carling *et al.* (2002a). Distance matrix values by the neighbour joining method were constructed and distances in the rDNA ITS region calculated using Kimura's two parameter model with sites with gaps omitted. Bootstrap analysis was performed with 1000 resamples of data. In all trees the rDNA ITS sequence of an AG1-IA isolate of rice (GenBank accession No. AB122133) was used as an out group.

5.3 Results

Nineteen isolates were sequenced in total, representing AG2-1, AG3, AG5 and AG8. Successfully sequenced isolates are shown in Table 5.1. Phylogenetic trees were constructed for isolates of AG2-1, AG3, AG4, AG5 and AG8. All sequences available on GenBank (as of November 2004) were included in the analysis except for trees constructed for AG3 and AG4, where due to the amount of sequence data present, sequences were selected for analysis based on host and country of origin.

Table 5.1 Isolates from which the rDNA ITS sequences were obtained

AG	Isolate	Host	Country of origin	GenBank Accession number
AG2-1 'l'	R22	Unknown	USA	
AG2-1 'l'	R42	Cauliflower	Netherlands	
AG2-1 'n'	X46	Potato	UK	
AG2-1 's'	X81	Potato	UK	
AG2-1 's'	Y2	Potato	UK	
AG2-1 'n'	Y3	Potato	UK	
AG2-1 'n'	Y25	Potato	UK	
AG2-1 's'	Y63	Potato	UK	
AG2-1 's'	Z1	Potato	UK	
AG3	UN	Potato	UK	
AG3	Y29	Potato	UK	
AG3	X56	Potato	UK	
AG3	X34	Potato	UK	
AG5	R48	Potato	France	
AG5	R52	Soybean	Japan	
AG5	T1	Couch grass	UK	AY545999
AG5	Y55	Potato	UK	
AG8	R28	Barley	UK	

Estimates of phylogenetic relationships for AG2-1 isolates are illustrated in Figure 5.3. Isolates from tulip clustered with isolates from crucifer and other plants except potato. Some potato isolates clustered with the tobacco isolate (AG2-Nt) present. The four isolates of AG2-1 's' clustered together, whilst isolates of the other AG2-1 'n' and 'l' did not. Branch lengths, especially with

isolates in the tulip and crucifer cluster varied, indicating a heterogeneous group.

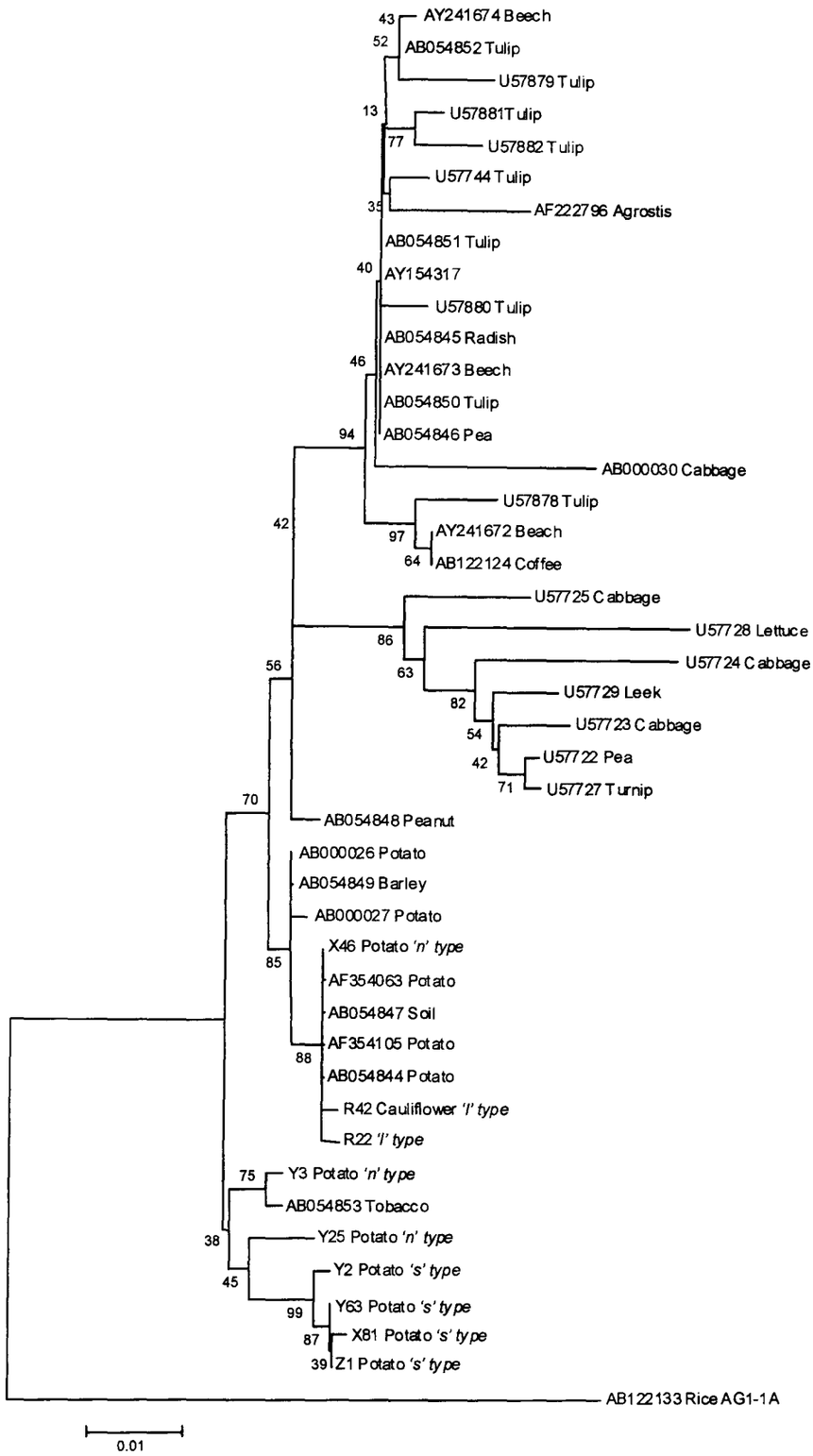


Figure 5.3 Neighbour joining tree illustrating phylogenetic relationships of the rDNA ITS region of AG2-1 isolates. Bar indicates one base change per 100 nucleotide positions. Numbers on branches are percentage of congruent clusters in 1000 bootstrap trials. GenBank accession number or isolate number given where appropriate. IGS1 types shown in italics where known.

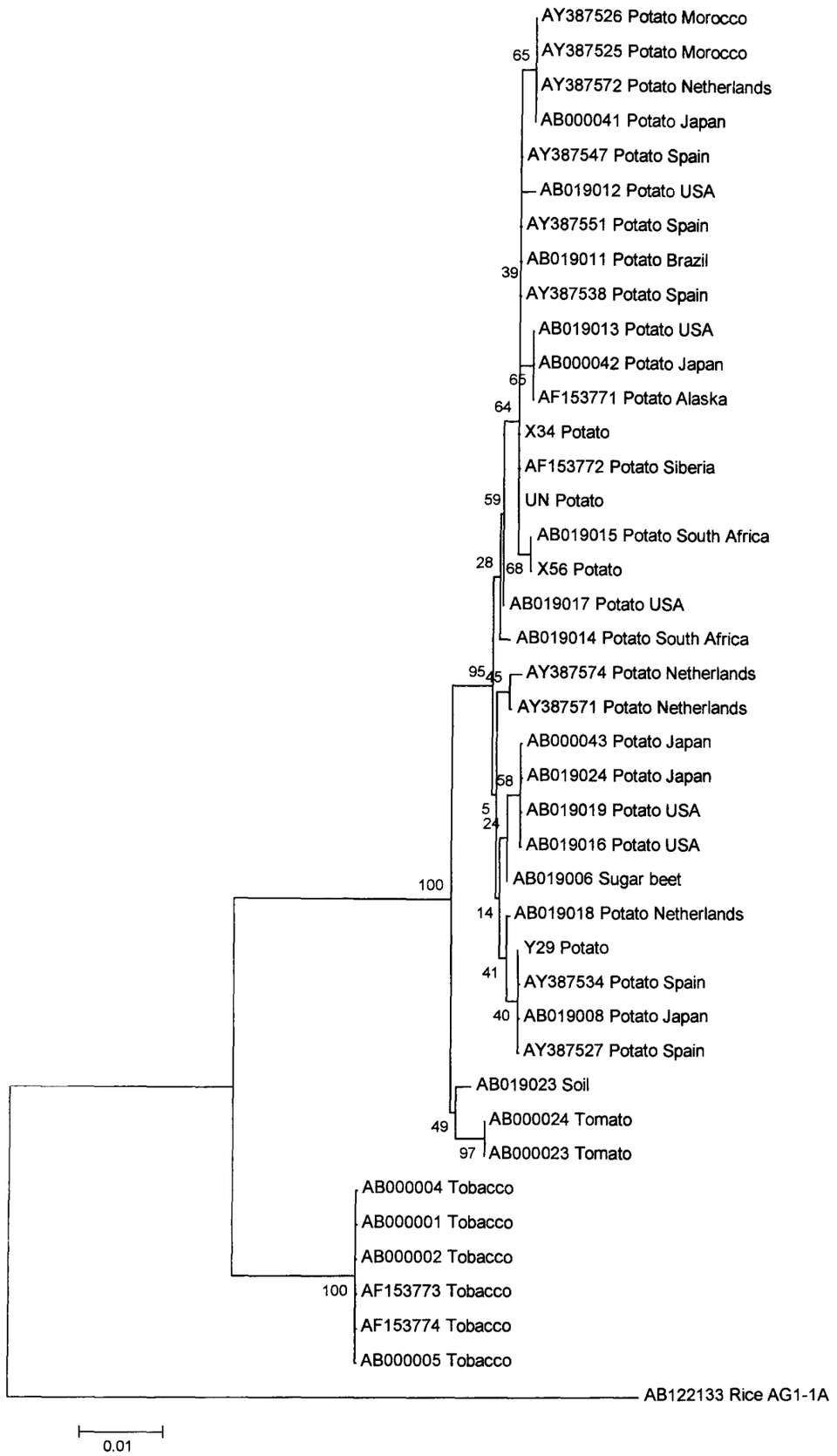


Figure 5.4 Neighbour joining tree illustrating phylogenetic relationships of the rDNA ITS region of AG3 isolates. Bar indicates one base change per 100 nucleotide positions. Numbers on branches are percentage of congruent clusters in 1000 bootstrap trials. GenBank accession number or isolate number given where appropriate.

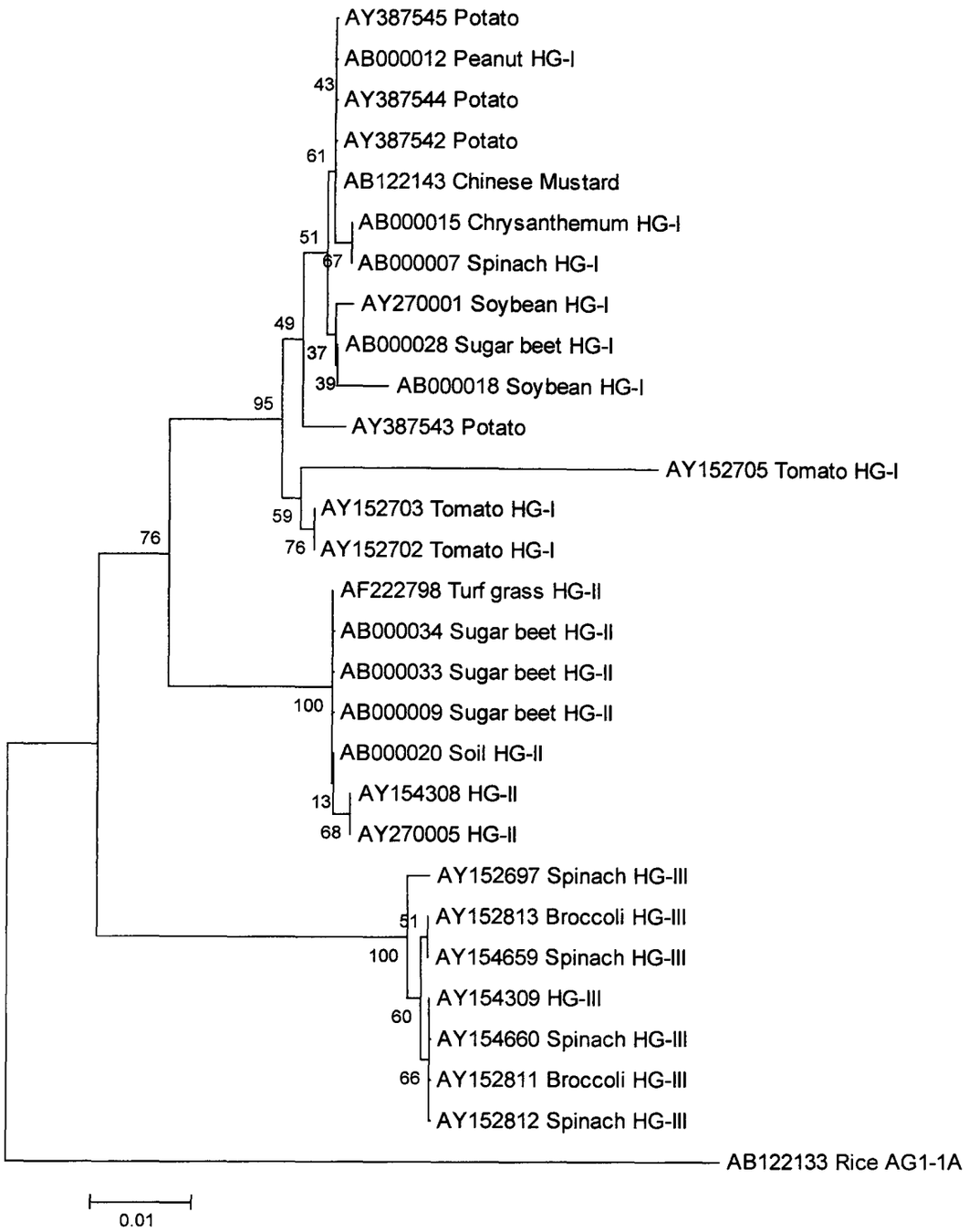


Figure 5.5 Neighbour joining tree illustrating phylogenetic relationships of the rDNA ITS region of AG4 isolates. Bar indicates one base change per 100 nucleotide positions. Numbers on branches are percentage of congruent clusters in 1000 bootstrap trials. GenBank accession number or isolate number given where appropriate.

Figure 5.4 illustrates phylogenetic relationships for isolates of AG3. Isolates from tobacco and potato plants formed separate clusters. Bootstrap values of 100% indicated a well defined grouping. A possible third subgroup of AG3 maybe present, consisting of the two tomato isolates and the one soil isolate.

One isolate of AG3 from sugar beet was clustered with the potato isolates, branch lengths indicated a homogenous group. Potato isolates from different countries were not clustered together.

Figure 5.5 supports the existence of the three subgroups already present in AG4 (HG-I to HG-III). Branch lengths and hosts indicate that HG-I is a diverse group; all potato isolates present on GenBank belonged to HG-I. Clustering suggested that isolates of HG-I are more related to HG-II than to HG-III. Relationships for AG5 isolates are shown in Figure 5.6. No clustering of isolates associated with host or country was observed. The two isolates from this study, both originating from Shropshire (Y55 and T1) did not appear to be closely related when compared to the sequences of other AG5 isolates.

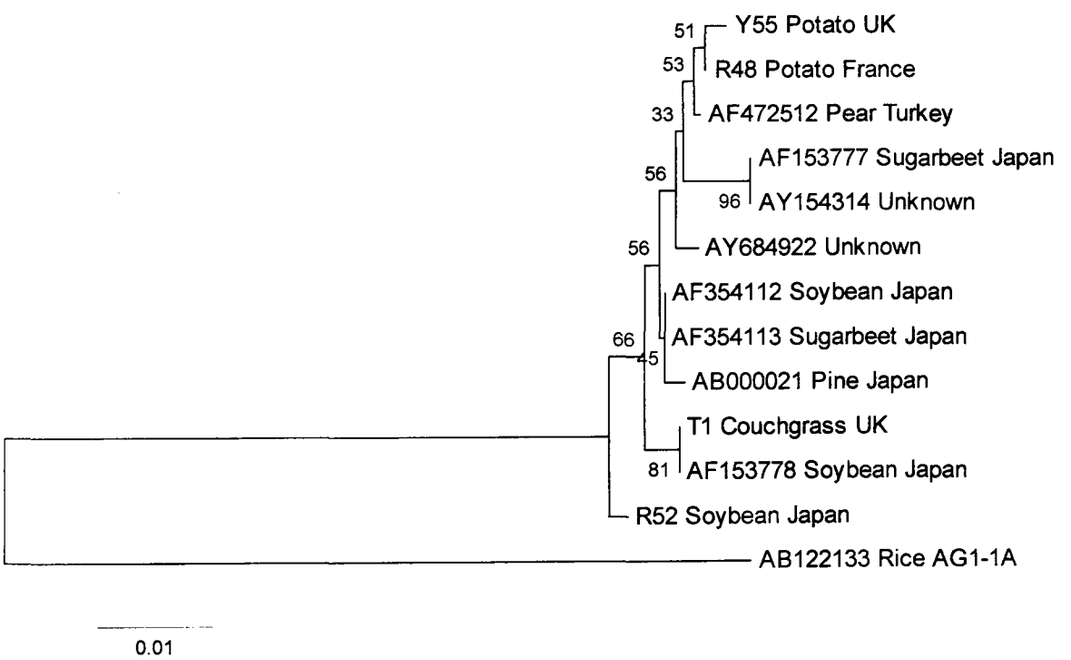


Figure 5.6 Neighbour joining tree illustrating phylogenetic relationships of the rDNA ITS region of AG5 isolates. Bar indicates one base change per 100 nucleotide positions. Numbers on branches are percentage of congruent clusters in 1000 bootstrap trials. GenBank accession number or isolate number given where appropriate.

Figure 5.7 illustrates the relationships for isolates of AG8. No clustering of the pectic zymogram groups was observed, however isolates from barley did cluster but with low bootstrap values.

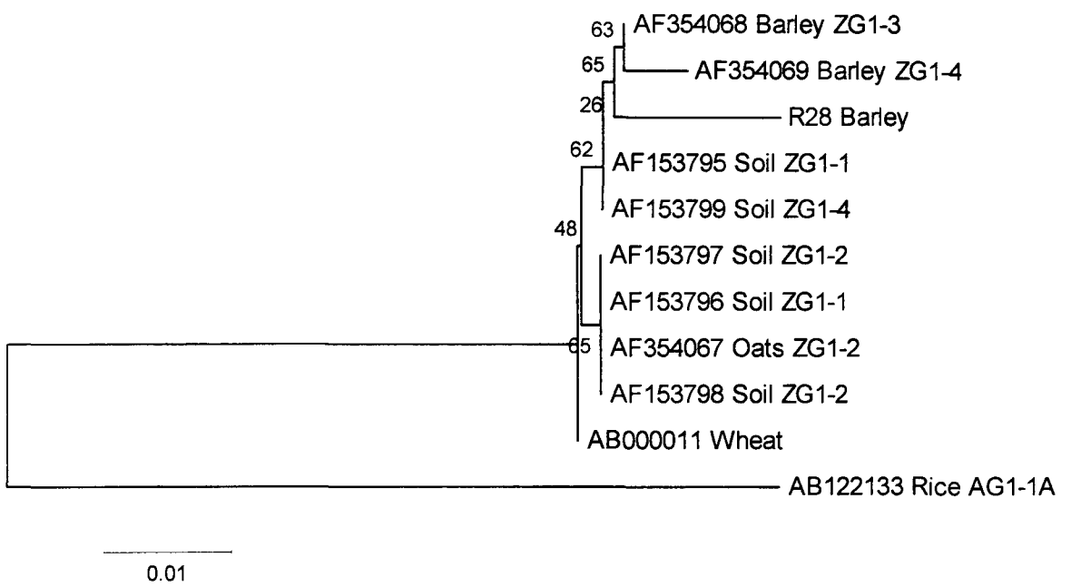


Figure 5.7 Neighbour joining tree illustrating phylogenetic relationships of the rDNA ITS region of AG8 isolates. Bar indicates one base change per 100 nucleotide positions. Numbers on branches are percentage of congruent clusters in 1000 bootstrap trials. GenBank accession number or isolate number given where appropriate.

5.4 Discussion

Figures 5.3 to 5.7 illustrate phylogenetic relationships amongst isolates of anastomosis groups associated with potato. Amongst isolates AG2-1, the tree in Figure 5.3 indicated that diversity was present. Previous studies involving phylogenetic analysis of AG2-1 rDNA ITS sequences have also observed diversity (Gonzalez *et al.*, 2001; Carling *et al.*, 2002a). Isolates of AG2-1 have often been assigned to subgroups on the basis of host and virulence. For example isolates from tulip and tobacco have been assigned AG2-t (Schneider *et al.*, 1997a) and AG2-Nt respectively (Nicoletti *et al.*, 1999). More recent studies have provided evidence for the inclusion of these subgroups into AG2-1 (Carling *et al.*, 2002a; Kuninaga *et al.*, 2000b). This study supports the inclusion, as the rDNA ITS sequences of tulip isolates were similar to those from other hosts including crucifers, beech, pea and coffee.

Previous studies did not include isolates of AG2-Nt within AG2-1 as hyphal fusion frequency between AG2-Nt isolates and AG2-1 isolates was low (Nicoletti *et al.*, 1999) or fatty acid analysis supported segregation (Priyatmojo *et al.*, 2002). The tobacco isolates of AG2 (AG2-Nt), clustered with several AG2-1 potato isolates in this study supporting the inclusion of AG2 Nt in AG2-1. Isolates of AG2-1 in this study were also assigned to IGS1 type. The rDNA sequences from the 'l' and 'n' IGS1 types did not cluster together, however isolates assigned to the 's' type did cluster, perhaps suggesting this is a subgroup of AG2-1. The 'n' and 'l' types may therefore belong to the same group, since the difference in IGS1 length between those

types is slight (approximately 20 bp), compared to the difference between the 's' and 'n' types. The difference between 'n' and 'l' types could therefore possibly be variation occurring in that group, it was observed that 'n' and 'l' isolates also resemble each other morphologically on potato dextrose agar, the 's' type differs in its sclerotia morphology and its ability to produce more aerial mycelia than the other types.

Previous studies have determined that isolates of AG2-1 can belong to one of the pectic zymogram groups ZG5 and ZG6 (MacNish *et al.*, 1994). It could be that these correspond well with the IGS1 types or the clusters in Figure 5.4, however no data is available. Further work is needed to determine the significance and implications of diversity in AG2-1, for example specific strains may have evolved a high level of specificity to certain hosts and consequently are unable to cause severe disease on other hosts. Such information would prove useful in the design of diagnostic assays and assessing disease risk.

Figure 5.5 supported the segregation of AG3 isolates of tobacco and potato into separate strains AG3PT and AG3TB. Previous studies have also shown good evidence for the segregation of AG3PT and AG3TB using analysis of rDNA ITS (Ceresini *et al.*, 1999; Kuninaga *et al.*, 2000a), rDNA IGS (Ceresini *et al.*, 1999) and AFLP analysis (Ceresini *et al.*, 2002b). This analysis used a larger number of isolates representing a greater geographical range but no clustering occurred amongst isolates of the same geographical origin. This suggests, with the short branch lengths in the tree, that AG3 is a

homogenous group. One isolate clustered with the AG3PT group was from sugar beet, indicating that AG3PT can survive on sugar beet, which may have important implications in crop rotation strategies.

The tree also indicated that a third subgroup of AG3 may exist. The two isolates from tomato and one from soil were in a separate cluster, but were more closely related to AG3PT isolates than AG3TB, this has also been observed previously (Kuninaga *et al.*, 2000a). This may be because both tomato and potato are closely related. However, before the existence of a third subgroup can be confirmed, more tomato isolates of AG3 need to be analysed not only with rDNA ITS sequences but also for differences in virulence between potato and tomato.

Tomato isolates also formed a separate cluster in Figure 5.5 within the HG-I subgroup of AG4. Here potato isolates of AG4 all belonged to HG-I, this may have implications for the design of diagnostic assays and control strategies. For example, hosts that AG4 HG-II and HG-III propagate on, need not be avoided in potato growing fields. Previous studies also have stated that pencycuron sensitivity/insensitivity is likely to be associated with the subgroupings of AG4 (Kim *et al.,* 1996), therefore if AG4 HG-I isolates are sensitive to pencycuron, then the use of the fungicide is likely to be effective.

All three subgroups of AG4 were also clearly in separate clusters, indicating well defined subgroups. Analysis of rDNA ITS sequences have confirmed the existence of the three subgroups in AG4, concordant with previous work

comparing fewer rDNA ITS sequences (Boysen *et al.,* 1996) or fatty acid composition (Stevens-Johnk and Jones, 2001).

Figure 5.6 indicated that AG5 is a relatively homogenous group. Despite this the two isolates which were both found in Shropshire, Y55 and T1 are not closely related in the context of other AG5 isolates. However, further sequence data is needed for this group as only 8 sequences were available on GenBank (November 2004). Sequence data for AG8 isolates is also limited in availability, all but one of the nine sequences available on GenBank are Australian in origin (the other is of UK origin). Isolates from barley did cluster, although isolates belonging to the same pectic zymogram group (MacNish *et al.,* 1994) did not cluster together. This suggests that isolates of different pectic zymogram groups are not genetically segregated, however a number of genes from a larger set of isolates need to be analysed before this can be confirmed.

To correctly infer phylogenetic relationships, a range of sequence data should be used, for example mitochondrial small subunit rDNA and β-tubulin sequences. This is because in some fungi evidence exists for divergent intragenomic rDNA ITS types that may lead to misleading conclusions (O'Donnell and Cigelnik, 1997). Analysis of the rDNA ITS sequences can prove useful in highlighting relationships between evolutionary units and assigning isolates to those units. In this instance, analysis of rDNA ITS sequences was used successfully to assign isolates to subgroup and support the existence of subgroups within an AG. The existence of subgroups within an

AG may have implications for the design of diagnostic tools and implementation of disease management strategies where particular differences are known to exist (e.g. in fungicide sensitivity or virulence).

6. Development of a PCR diagnostic assay for *Rhizoctonia solani* AG3PT

6.1 Introduction

The techniques used to determine AG based on the observation of hyphal fusion are laborious, slow and prone to misinterpretation due to compatibility between certain AGs and incompatibility within an AG (Table 2.2). Therefore, there is a present need for a more standardised, robust method of identifying the AG of an isolate. Also, methods for the detection and quantification of the fungus in plant and soil material are not only useful research tools but can also be used to monitor disease in crops, predict outbreaks and allow the implementation of an early control strategy.

Several different techniques have been developed which allow the identification and/or detection of *R. solani*. The earliest of these techniques involved the isolation of the pathogen from the soil using selective media such as that described by Ko and Hora (1971) and Trujillo *et al.* (1987). The size of the *Rhizoctonia* population could be estimated by the number of colony forming units present after a period of incubation. However, these methods could not determine which AGs were present. Castro *et al.* (1988) adapted the selective media technique to allow quantitative estimation of AG3 in soil. This involved examining colonies microscopically and identifying and transferring *Rhizoctonia* colonies onto Stewart's medium, which can distinguish AG3 by the presence of white colonies compared to the brown colonies seen for other AGs (Castro *et al.*, 1983). These techniques are labour intensive, cumbersome

and require taxonomic expertise (Thornton *et al.,* 1993), therefore recent research has concentrated on immunological and molecular methods.

6.1.1 Immunological assays for detection of *Rhizoctonia solani*

Several immunological based methods have been developed for the detection of *R. solani.* Dusunceli and Fox (1992) used a monoclonal ELISA (enzyme linked immunosorbent assay) to detect *R. solani* in soil. Similarly, Thornton *et al.* (2004) developed an immunochromatographic lateral flow device to detect and quantify *R. solani* in soil. However, both these tests lack specificity to *R. solani*, detecting other *Rhizoctonia* species and not distinguishing between AGs.

An AG specific immunological assay to identify AG3 sclerotia on potato tubers was developed by Hingland and Lecozgillet (1993). Similarly, Miller *et al.* (1992) developed an assay specific for AG2-2, but this cross-reacted with *Ceratobasidium cornigerum* (then known as *R. cerealis*). Despite the occurrence of cross-reactions, immunoassays have the potential to distinguish between proteins that differ by just one amino acid residue (Kleinsmith and Kish, 1995) and this suggests that the development of AG specific assays should eventually be possible. Presently, the design of such specific immunoassays is difficult and consequently many AG specific assays available are based on nucleic acid sequences.

6.1.2 Nucleic acid assays for detection of *Rhizoctonia solani*

Individual anastomosis groups can be identified and detected using a variety of techniques based on differences in nucleic acid sequence. Such techniques offer the advantage of being highly specific, sensitive and not affected by environment or stages in the life cycle of the fungus.

Techniques able to distinguish individual AGs include Restriction Fragment Length Polymorphisms (RFLP) (Schneider *et al.,* 1997b), fingerprinting techniques such as universally primed-PCR (Lübeck and Poulson, 2001) and RAPD-PCR (Duncan *et al.,* 1993; Yang *et al.,* 1995; Pascual *et al.,* 2000). However these methods have limitations, as DNA must be extracted from isolates of *R. solani* and therefore they cannot be used to detect AGs directly in plant or soil material. DNA probes that can determine the presence of individual AGs in plants and soil have been developed. Probes have been developed for AG3 (Jabaji-Hare *et al.,* 1990; Balali *et al.*, 1996) and AG8 (Matthew *et al.,* 1995; Whisson *et al.,* 1995).

The discovery of the polymerase chain reaction (PCR) has largely replaced the use of such probes. PCR shares many of the advantages of DNA probes including sensitivity and is also less time consuming and safer as no autoradioactivity is required. Since the discovery of PCR in the mid 1980s, it has revolutionised molecular biology (Mullis *et al.,* 1994) and has emerged as a major tool for the diagnosis and study of phytopathogenic fungi, allowing

some of the problems associated with the detection, control and containment of plant pathogens to be overcome (Martin *et al.,* 2000).

PCR allows the amplification of specific DNA fragments and is carried out by combining the target DNA molecule with deoxynucleotide triphosphates (dNTPs), two synthetic oligonucleotide primers and a thermostable DNA polymerase. Primers are short single stranded DNA sequences that bind to the target DNA through complementary base pairing and determine the specificity of the reaction. Heating the mixture to around 95°C separates the two strands, the temperature is then reduced to between 40 and 65°C to allow the annealing of the two primers at either side of the specific region to be amplified. The temperature is then increased (usually to 72°C) to allow the polymerase to extend from the primers, effectively copying the single stranded target DNA. This is repeated 35 to 40 times until millions of copies of the sequences have been produced. The presence of the amplified product is checked by agarose gel electrophoresis; absence of amplified product of the expected size (bp) means that the target DNA for which the primers were designed for was not present or that a false negative has occurred. False negatives can be attributed to reaction failure, possibly due to inhibitors in the sample or user error. The use of positive and negative controls and internal standards can be used to indicate erroneous results.

PCR assays have also been used for the quantification of pathogen DNA. Currently, two quantitative PCR assays are used predominantly (there are others): competitive PCR and real-time PCR. Competitive PCR involves the

construction of an internal standard that has identical primer sites as the target DNA. The internal standard is of a different size allowing the target and internal standard amplicons to be distinguishable after electrophoresis. The internal standard is added to each PCR at a fixed concentration and amplified under the same conditions as the target DNA, at the end of the reaction the ratio of the two products is proportional to the concentration of target DNA present at the start of the reaction. Amount of target DNA is determined by comparing band intensities with known target DNA concentrations.

Real-time PCR is an alternative approach to quantitative PCR and despite the apparatus required for the assay being significantly more expensive than that needed for competitive PCR, it offers several advantages. For example, it has greater sensitivity and no post-PCR handling (i.e. electrophoresis), thereby increasing sensitivity and reducing potential errors, cross contamination, time and labour (Edwards *et al.,* 2002).

Real-time PCR uses labelled probes, detected by an inbuilt detector for florescent DNA probes specific to the target DNA or alternatively non-specific DNA binding dyes (e.g. SYBR Green), this means post PCR handling is avoided. The higher the concentration of target DNA present in a particular sample, the quicker the PCR reaction enters the exponential (log) phase of amplification. The amount of PCR product amplified is measured during each PCR cycle by the automatic detection of the florescent dyes or probes by the apparatus, and a cycle threshold (CT) is calculated. The cycle threshold is the cycle number at which fluorescence (hence product) becomes measurable.

The cycle threshold therefore increases with decreasing amounts of target DNA. Comparison of the CT value generated by an individual unknown sample with those of a standard curve generated with known concentrations of target DNA allows the concentration of the target DNA to be determined (Edwards *et al.,* 2002; Schena *et al.,* 2004; Ward *et al.,* 2004).

Several tests based on PCR have been used to detect *Rhizoctonia* and primers were often designed from previously sequenced genes such as the well-characterised rDNA ITS sequences. Such assays have been used to identify subgroups of AG1 (Matsumoto, 2002), AG2 (Carling *et al.,* 2002a; Matsumoto, 2002) and AG3 (Kuninaga *et al.,* 2000a) in pure culture. PCR has also been used to detect the presence of *R. solani* in plant material. Salazar *et al.* (2000b) designed primers able to detect various AG2 subgroups and binucleate *Rhizoctonia* in plant tissue. Toda *et al.* (1999) designed specific primers to detect AG2-2LP in the leaf sheaths of zoysia grass (*Zoysia matrella).* DNA sequences other than the rDNA region have been used as target sequences for AG specific primers. Brisbane *et al.* (1995) used RAPD-PCR to obtain sequence tagged site markers in the design of specific primer sets. These primers were used to detect the presence of AG4 and AG8 in wheat.

The detection of individual AGs in the soil is more difficult, due to the presence of PCR inhibiting compounds often co-extracted with DNA from soil. Despite such difficulties, a conventional PCR assay for the detection of AG3 has been developed (Bounou *et al.,* 1999). More recently Lees *et al.*

(2002) developed a quantitative real-time PCR assay for the detection of AG3 in soil. However detection was found to be variable, possibly due to the patchy distribution of the pathogen in soil and/or due to PCR-inhibitory compounds. This chapter describes the development of a competitive PCR assay to detect the presence of *R. solani* AG3PT.

6.2 Materials and methods

6.2.1 Primer design and optimisation of the PCR assay for AG3PT

Putative AG3PT specific primers were designed from the internal transcribed spacer regions (ITS1 and 2) of ribosomal DNA (rDNA). ITS sequence data for isolates of *Rhizoctonia* were accessed on the GenBank nucleotide database. ITS sequence data from Chapter 5 was also utilised. Using BioEdit (Hall, 1999), consensus sequences were constructed for 23 *R. solani* isolates representing AGs 1 to 12. A consensus sequence was also constructed for *C. cornigerum* (≡ *R. cerealis*). These sequences were aligned using ClustalW (Thompson *et al.,* 1994) and potential primers were designed from regions of sequence unique to AG3PT.

In order to determine the optimum conditions for the diagnostic assay, PCR was performed using the primer set with isolates of AG3PT and isolates of closely related AGs. PCR was performed simultaneously at two different annealing temperatures using PCR reagents and sample DNA from the same stock. The annealing temperature was increased until no cross-reaction was present and only products from AG3PT sample DNA were present. This annealing temperature was used in all subsequent PCRs.

6.2.2 DNA extraction from plant material

DNA was extracted from potato leaves, stems, roots and tubers. Each type of tissue was prepared by washing under tap water, then surface sterilising the appropriate section with sodium hypochlorite solution (1% available chlorine) and two rinses of SDW. One gram of potato material was excised using a scalpel and heat-sealed in an acetate envelope and subsequently crushed using a hammer. DNA was extracted from crushed samples using a cetryltrimethylammonium bromide (CTAB) buffer (sorbitol, 23g; N-lauryl sarcosine, 10 g; CTAB, 8 g; sodium chloride, 87.7 g; EDTA, 8 g; polyvinylpolypyrrolidone, 10 g and water to 1 l). Five ml of CTAB buffer was added to the crushed potato material in a 30-ml tube, mixed and incubated at 65°C for 16 h. Two ml of 5M potassium acetate was added to each tube and samples were mixed and then frozen at –20°C for at least 1 h.

After thawing, the contents of each tuber were mixed again and centrifuged at 3000 x g for 15 min. Then 1.3 ml of supernatant was removed and added to 0.6 ml of chloroform in a 2-ml Eppendorf tube. Samples were mixed by gentle inversion for one minute followed by centrifugation at 12000 x g for 15 min. One ml was transferred from the aqueous phase of the sample into a tube containing 0.8 ml of 100% isopropanol. Samples were again gently mixed for one minute, incubated at room temperature for 30 min and centrifuged at 6000 x g for 15 minutes to form a DNA pellet. The pellets were then washed twice with 70% ethanol and left to air dry until no ethanol was visible. Pellets were re-suspended in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by

vigorous mixing and leaving overnight at room temperature. DNA was stored at 4°C for short-term storage or at –20°C for long-term storage. DNA was quantified by UV spectrophotometer as described in Chapter 2.

6.2.3 DNA extraction from soil

In order to determine the best method for the extraction of *Rhizoctonia* DNA from soil, four different methods were tested and are described here, each categorised by their main extraction ingredient. Using each method, three separate DNA extractions were performed from the same sample of *R. solani* AG3PT infected soil. The infected soil was prepared by inoculating 500 g of field soil (sandy loam) with 10 g macerated *R. solani* mycelia. The field soil originated from an area where potatoes had not been grown for eighteen years and therefore assumed to have little or no *R. solani* present prior to inoculation. The inoculated soil was thoroughly mixed and left for three days at room temperature to dry, after which it was mixed again, sieved (5 mm mesh) and subsampled appropriately for each assay. The subsamples were transferred to 30-ml plastic tubes and stored at -20°C until needed, when they were prepared for DNA extraction by freeze-drying. The concentration of DNA present in the samples was quantified and stored as described in Chapter 2.

6.2.3.1 Extraction of DNA from soil with CTAB buffer

This method was adapted from Lees *et al.* (2002). CTAB buffer was prepared as described previously and 20 ml was added to 10 g of freeze-dried soil, vortexed for 3 s and placed in an ultrasonic water bath (Branson, Shelton, USA) for 30 minutes and vortexed again for 3 s. Then 1 ml was transferred to a 2-ml Eppendorf tube containing 0.2 g sterile zirconia/silica 0.1 mm beads and 0.2 g sterile 1 mm glass beads. The lid was placed on the vial and placed in a soil mill (Griffin, UK) for one hour. The vial was then centrifuged at 3000 x g for 5 minutes and the supernatant transferred into a 2-ml Eppendorf tube containing 750 µl cold chloroform. The tube was shaken by hand for 30 s and centrifuged at 13000 x g for 5 minutes. The upper aqueous phase was transferred to a new tube containing an equal volume of isopropanol and left for one hour at room temperature. The tube was centrifuged at 13000 x g to pellet the DNA and the supernatant was removed. The pellets were air dried for 5 to 15 minutes and 100 µl TE added. The sample was purified through a spin filter (Costar, New York, USA) containing 700 µl pre-swollen Sephadex G-75 (Sigma) in TE by centrifuging at 2000 x g for 3 min. DNA was stored as described in Chapter 2.

6.2.3.2 Extraction of DNA from soil with skimmed milk powder

DNA was extracted from soil by a method using skimmed milk powder as described by Volossiouk *et al.* (1995). A solution was prepared by adding 0.1 g of skimmed milk powder to 25 ml of sterile distilled water. Twenty

millilitres of the milk powder solution was added to a 10 g freeze-dried soil sample, the sample was vortexed for 30 s and 1 ml was transferred to a 2-ml Eppendorf tube containing 0.2 g of sterile zirconia/silica 0.1 mm diameter beads and 0.2 g of sterile 1 mm diameter glass beads. The sample was vortexed for 30 s and incubated in a shaking 65°C water bath for one hour after which time 300 µl cell lysis solution (Puregene) was added and the sample was vortexed for 30 s. The sample was centrifuged at 12000 x g and the supernatant then transferred to a sterile tube containing 100 µl ammonium acetate (10M) and vortexed for 30 s. The sample was centrifuged at 12000 x g and the aqueous phase transferred to a fresh tube with 300 µl chloroform. The tube was inverted several times and centrifuged for 5 minutes at 13000 x g. The upper aqueous phase was transferred to a new tube containing an equal volume of isopropanol, inverted several times and centrifuged at 13000 x g to pellet the DNA. Pellets were rinsed with 70% ethanol, air dried and then 50 µl of TE was added to the dry pellet. DNA was re-suspended and stored as described in Chapter 2.

6.2.3.3 Extraction of DNA from soil using guanidinium thiocyanate buffer

This method, adapted from Towner (2000), uses guanidinium thiocyanate as a solubilisation buffer and diatomaceous earth as binding resin for DNA. Guanidinium thiocyanate solubilisation buffer was prepared by dissolving 472 g of guanidinium thiocyanate in 500 ml water, adding 50 ml of 1M Tris-HCl (pH 7.5) and 40 ml 0.5M EDTA (pH 8.0) and making up to 1 l. The DNA binding resin was prepared by suspending 10 g of diatomaceous earth in 2 l of

water, allowing the mixture to settle for one hour and pouring away the supernatant. The diatomaceous earth was then re-suspended in a further 2 l of water and allowed to settle for one hour before discarding the supernatant. The diatomaceous earth was re-suspended in guanidinium thiocyanate solubilisation buffer prepared as above except that the volume was restricted to 900 ml. After addition of the diatomaceous earth the buffer was made up to 1 l.

Freeze dried soil (0.25 g) was placed in a 2-ml Eppendorf tube containing 0.2 g of sterile zirconia/silica 0.1 mm diameter beads and 0.2 g sterile 1 mm diameter glass beads and 1 ml solubilisation buffer. The sample was vortexed gently and 60 µl of SDS solution (100mM Tris-HCl, pH 7.5, 100mM NaCl, 10% SDS) and 200 µl of ascorbic acid added. The sample was then horizontally vortexed at maximum speed for 10 minutes and centrifuged at 10000 x g for 30 s. The supernatant was transferred to a clean tube containing 250 µl 5M potassium acetate, vortexed for 5 s and then incubated a 4°C for 5 minutes. Samples were centrifuged for 1 minute at 10000 x g and avoiding the resulting pellet, the entire volume of supernatant was transferred to a clean 2-ml Eppendorf tube.

Then 1.3 ml of DNA binding resin (diatomaceous earth) was added and the sample was vortexed for 5 s. Approximately 700 µl of the sample was loaded into a Spin filter (Costar), centrifuged at 12000 x g for 1 minute and the flow through discarded. This was repeated until the entire sample had passed through the spin filter. Three hundred micro litres of ethanol wash solution

(50mM Tris-HCl, pH 7.5, 10mM EDTA, pH 8.0, 0.2M NaCl, 50% (v/v) ethanol) was then added to the spin filter and centrifuged at 12 000 x g for 30 s and the flow through discarded again. The spin filter was transferred to a clean tube and centrifuged at 10000 x g for one minute to dry the tube. Finally the DNA was re-suspended by transferring the spin filter to a clean tube and 50 µl of water was placed on the centre of the membrane. The water was left to absorb into the membrane for two minutes and the spin filter was centrifuged again at 13000 x g to collect the DNA solution. The spin filter was discarded and DNA was stored as described in Chapter 2.

6.2.3.4 Extraction of soil DNA using the Ultra Clean Soil DNA Kit

The Ultra Clean Soil DNA kit was supplied by MoBio Laboratories Incorporated, California, USA. DNA was extracted from soil according to the manufacturer's instructions.

6.2.4 Internal standard preparation

In order for the diagnostic test to be quantifiable, an internal standard for the primer pair Rs1F2 and AG3PR2 was constructed using the methods of Förster (1994). An internal standard (AG3PIS) was produced from the onion alliinase gene (EMBL accession No. L48614) as described by Edwards *et al.* (2001). A 1.2 Kb fragment from the alliinase gene was amplified using PCR (as Chapter

2) at an annealing temperature of 58°C with primers ONI/F (TGC TCT GCT GAT GTT GCC AG) and ONI/R (TAC ATG GGG ATG GAG GTC TC). After electrophoresis, the 1.2 Kb product was excised from the gel, and incubated in 1 ml of TE buffer at 4°C for at least 16 h. Five microlitres of this solution was then amplified with linker primers, AG3P/FL (TGG TCT ATT TGT TGC TCA TGC CCC) and AG3P/RL (CCA GCT AAT ATG AGG TCG CGC ATG), using a Touchup PCR programme which differed from that described in Chapter 2 in that it consisted of ten cycles of an anneal of 38°C, followed by 20 cycles at 55°C to produce a linker product which consisted of the onion alliinase gene segment bordered by 10 bp of the AG3PT primers either side.

The linker product was excised from the gel and incubated in 300 µl of TE buffer at 4°C for at least 16 h. Again 5 µl was removed from this solution for PCR using the Touchup programme to produce a 670 bp alliinase segment–primer site construct. This was removed from the gel and purified using a Wizard PCR Prep Kit (Promega, Southampton, UK). The purified product was ligated into a pGEM-T Vector (Promega) and transformed in *Escherichia coli* JM109 according to the manufacturer's instructions. After incubation, white colonies were screened for the internal standard using primers Rs1F2 (Lees *et al.*, 2002) and AG3PR2. Positive clones were selected and grown overnight in LB broth (Merck, Darmstadt, Germany), plasmid DNA was then extracted using the Wizard Plus SV Miniprep Kit (Promega). Internal standard plasmid DNA was then diluted ready for use with TE buffer.

PCR assays including the internal standards were performed as described in Chapter 2 except the total reaction consisted of 50 μl, including 10 μl of sample DNA and 10 μl internal standard plasmid DNA.

6.3 Results

6.3.1 Primer design and optimisation of the PCR assay for AG3PT

Five sets of primers with potential specificity for AG3PT were synthesised in total, including Rs1F2 and Rs2R1 as described by Lees *et al.* (2002). A product of the expected size was observed when DNA of AG3PT was amplified in a PCR assay with primers Rs1F2 and Rs2R1 at an annealing temperature of 65°C. Products were not observed in DNA from any other AG tested except DNA from AG2-1's' isolates. To prevent the cross reaction the annealing temperature was increased gradually and no cross-reaction was observed at an annealing temperature of 68°C, however at this temperature the amplification efficiency was low, resulting in a faint band when AG3PT DNA was amplified.

Subsequent sequencing of the AG2-1 's' isolates revealed that they do not possess a mismatch at the 3' end of the Rs1F2 primer (Figure 6.1). Primer Rs2R1 was also either identical or very similar (possessing just a 1 bp mismatch at the 9th base) with the sequence of the AG2-1 's' isolates. To overcome the cross reaction the primer AG3PR2 (ACA CTG AGA TCC AGC TAA TA) was designed that possessed a 1 bp mismatch at the 3' end for AG2-1 's' isolates and isolates of the majority of other AGs (Figure 6.2). The use of primers AG3PR2 and Rs1F2 as a pair produced a 474 bp sized product only in AG3PT isolates at an annealing temperature of 65°C. This primer pair was used was through out this study to confirm the identity of AG3PT isolates.

Rs1F2	T	T	G	G	T	T	G	T	A	G	C	T	G	G	T	C	T	A	T	T	T
AG1-IA	.	G	A	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	.	C
AG1-IB	.	G	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	A
AG1-IC	.	G	A	.	.	.	.	.	T	.	.	.	.	.	C	.	.	C	.	G	G
AG2-1n	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	C
AG2-1's'	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.
AG2-2IIIB	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	C	A	.	.
AG2-2IV	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	C	A	.	.
AG2-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	C	G
AG2-BI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A
AG3PT	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG3TB	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	A
AG4 HGI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	A	A
AG4 HGII	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	C	.	A	A
AG5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	C	A
AG6 HG1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	G	A	.	.
AG6 GV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	A	.	.
AG7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	G	A	.
AG8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A
AG9 TP	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C
AG9TX	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C
AG10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A
AG11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	.	.	.	A
AG12	.	.	G	G	.	.	T	.	C	T	T	.	.	.	G	.	A	T	G	.	G
C. cornigerum	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	T	C	T	.	.	.

Figure 6.1 Alignment of primer RS1F2 with consensus sequences from 24 other *Rhizoctonia* subgroups

AG3PR2 (inverted)	T	A	T	T	A	G	C	T	G	G	A	T	C	T	C	A	G	T	G	T
AG1-IA	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG1-IB	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG1-IC	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-1n	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-1 's'	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-2IIIB	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-2IV	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG2-BI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG3PT	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG3TB	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG4 HGI	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG4 HGII	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG5	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG6 HG1	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG6 GV	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG7	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG8	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG9 TP	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG9TX	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG10	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AG12	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TCG	.	.	.	.
C. cornigerum	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	A	A

Figure 6.2 Alignment of primer AG3PR2 with consensus sequences from 24 other *Rhizoctonia* subgroups

6.3.2 Validation of the diagnostic PCR assay for AG3PT

In order to validate the assay, the primer pair AG3PR2 and Rs1F2 was tested on a wide selection of isolates of AG3PT, other AGs and other potato pathogens (Table 6.1). Thirty isolates of *R. solani* gathered in 2001 (Chapter 3) were confirmed to be AG3PT by observation of hyphal fusion with isolate UN. Isolate UN itself was confirmed to be AG3PT through ITS sequence comparison and hyphal fusion with previously determined AG3PT isolates R36, I2, I3, I4 and I5. All isolates of AG3PT were amplified by AG3PR2 and Rs1F2 to give a 474 bp product. All other isolates tested were not amplified. Plant and soil material (see Table 6.1) containing AG3PT also gave positive results with this primer pair.

Table 6. 1 Material used to validate the PCR assay for AG3PT

Species	Form	Isolate/material used
Rhizoctonia solani AG3PT	Pure culture	30 AG3PT isolates collected in 2001 plus UN, R36, Y29, I3
	Infected stem	Infected by UN, R36, Y29, I3
	Sclerotia on tuber	UN, R36, Y29
	Infected roots	UN, R36, Y29
	Infested soil	UN, Y29
Rhizoctonia solani AG2-1	Sclerotia on tuber	X81
	Stems displaying stem lesion	X81, X52, Y2, Y3
	Pure culture	AG2-1 isolates in Table 2.1
Rhizoctonia solani AG1 to 8	Pure culture	Isolates listed in Table 2.1
Ceratobasidium cornigerum (≡R. cerealis)	Pure culture	130/2SE*
Phytophthora infestans	Pure culture	A1 isolate 95.9.5.1* A2 isolate 97.9.1.1*
Helminthosporium solani	Tuber blemish	Section of tuber infected with silver scurf
Streptomyces scabies	Tuber blemish	Section of tuber infected with common scab
Uninfected material	Potato	Stems, leaves, tuber, root
	Soil	Field soil and compost
Cracking symptoms of unknown cause	Tuber	Three tuber samples from different sites

*From the Harper Adams University College culture collection

6.3.3 DNA extraction from soil

The yield and purity of the DNA obtained from the soil extraction methods tested are given in Table 6.2. DNA extracted from soil was not as pure as DNA extracted from pure culture (A_{260}/A_{280} = 1.8 for pure DNA). Soil DNA extraction using the Ultra Clean Soil DNA kit produced the purest DNA with purity values of 1.72 and 1.75. Most other samples extracted from soil had purity value of 1.26 to 1.5, suggesting contamination by protein. The exception being two samples extracted with guanidinium thiocyanate buffer that had purity values above 2.2 which suggests the co-extraction of other contaminants.

Table 6. 2 Comparison of four soil DNA extraction methods and PCR result.

Method	Replicate	A_{260}	A_{280}	A_{328}	A_{360}	Purity[1]	Concentration ($\mu g\ \mu l^{-1}$)	PCR product
CTAB	1	0.088	0.080	0.063	0.055	1.49	98.31	Yes
	2	0.084	0.067	0.034	0.021	1.50	194.26	Yes
	3	0.060	0.054	0.031	0.021	1.26	100.64	No
Skimmed milk	1	0.010	0.007	0.001	-0.003	1.44	36.55	No
	2	0.042	0.035	0.017	0.010	1.42	93.69	No
	3	0.034	0.028	0.013	0.008	1.47	80.46	No
Guanidinium thiocyanate	1	0.026	0.012	0.002	-0.003	2.24	116.02	Yes
	2	0.089	0.071	0.034	0.015	1.47	212.87	Yes
	3	0.014	0.005	-0.001	-0.005	2.32	73.90	No
Ultra Clean Soil DNA kit	1	0.036	0.024	0.007	-0.005	1.72	120.85	Yes
	2	0.041	0.029	0.010	-0.005	1.75	129.84	Yes
AG3PT culture[2]	1	0.307	0.208	0.086	0.056	1.81	949.82	Yes
	2	0.474	0.329	0.157	0.106	1.84	1372.82	Yes

[1]Purity determined as in Chapter 2; [2]DNA extracted from UN (1) and Y29 (2) hyphal cultures as described in Chapter 2.

Suitability of the soil DNA samples for PCR were tested using primers AG3PR2 and Rs1F2 with an annealing temperature of 65°C. Originally, PCR products were not produced by all samples listed in Table 6.2, therefore to improve efficiency of PCR with DNA samples from soil, putative PCR enhancing additives were tried, including skimmed milk powder (Marvel) and

bovine serum albumin (BSA). It was found that BSA (0.1 mg ml^{-1}) improved the efficiency of the PCR with no cross-reaction with other AGs. Skimmed milk powder (1 μg μl^{-1}) was also found to improve efficiency. However skimmed milk powder solution needed to be made up fresh before every reaction, making the use of BSA easier. The presence of skimmed milk powder and BSA together did not improve efficiency noticeably compared to their use individually.

The results presented in Table 6.2 were generated with the addition of BSA in the PCR assay. No PCR product was amplified from DNA extracted from soil using the skimmed milk method. PCR was successful with two of the three DNA samples extracted using CTAB or guanidinium thiocyanate buffer. PCR was also successful with both DNA samples extracted using the Ultra Clean Soil DNA kit; each of these samples had a DNA purity ratio close to 1.8.

6.3.4 Quantification of *Rhizoctonia solani* in soil

PCR was performed using the soil sample as described above, with the addition of the internal standard at approximately 5 to 20 fg μl^{-1} (in increments of 5). No AG3PT PCR product was observed with the addition of the internal standard at any of these concentrations, however internal standard DNA was visualised. This was because the quantity of AG3PT DNA extracted was very low, and consequently the addition of the internal standard out competed the AG3PT DNA present. Internal standard was not used below 5 fg μl^{-1} due to the difficulty of producing a consistent standard curve at such concentrations.

6.4 Discussion

In this study a diagnostic assay that can specifically detect DNA of *R.solani* AG3PT in plant tissue and soil, as well as from pure culture, was developed. Primers were designed that appear to be more specific than those previously available, which were shown to cross-react with isolates belonging to a prospective AG2-1 subgroup, AG2-1 's'. The ability of the assay to discriminate between AG2-1 's' and AG3PT is important because of the profound differences in disease development and host range between the groups. Despite AG3TB and also AGs 9 to 12 not being tested with primers AG3PR2 and Rs1F2, differences between the sequence of those primers and AG3TB and AGs 9 to 12 are more different than that of AG2-1s, suggesting this assay would discriminate against AG3TB and AGs 9 to 12.

Discrimination between AG3PT and AG3TB could possibly be important in areas where potato and tobacco are both grown as the subgroups have a host range that does not overlap, both subgroups having evolved a high level of specificity on their different solanaceous hosts (Ceresini *et al.,* 2002b).

DNA of AG3PT could be detected by PCR using DNA extracted from soil using several different methods in this assay. The Ultra clean soil DNA kit was the best method as the DNA extracted was the purist, however the guanidinium thiocyanate method showed potential. The guanidinium thiocyanate method offers the advantage of possibly being suitable for high throughput use as the method does not require DNA pelleting, unlike the other

methods. DNA pelleting and drying are time consuming and loss of the pellet can occur.

Despite the ability of the test to detect AG3PT in the soil, with the addition of the internal standard, the amount of *R. solani* present could not be quantified unlike the results of Lees *et al.* (2002). This is likely to be because very low levels of target DNA were extracted from the soil and were co-extracted with PCR-inhibitory compounds. This was common to all of the methods of DNA extraction and further work needs to be done to optimise the extraction technique to extract more DNA and/or extract less inhibitors.

This suggests that the DNA of *R. solani* extracted from soil in this experiment was too low to be quantified using competitive PCR. The PCR assay of Lees *et al.* (2002) was based on real-time technologies, which are more sensitive than competitive PCR (Schena *et al.,* 2004). In order to improve this assay then it would have to be adapted for use with real-time PCR technology or the DNA extraction method optimised further.

Alternatively, a baiting step could be incorporated into the test, this would increase the amount of *R. solani* DNA present, this is termed BIO-PCR. DNA could then be extracted from the baits, as opposed to directly from soil, and quantified. This method was successfully demonstrated by Lees *et al.* (2002), using quinoa seeds incubated in infested soil to increase the amount of AG3 target DNA present. Beetroot seeds are also considered suitable baiting material for *R. solani* (Kyritsis and Wale, 2002c) and could also be used.

Adding a baiting step would increase the time taken to do the assay. However it does offer the advantage of detecting only viable infectious units. Quantitative PCR direct from soil may be misleading as there could be an inconsistent relationship between DNA concentration and infection potential of *R. solani* in soil due to the different fungal structures present and their physiological state

Further work would involve developing this assay to quantify AG3PT in soil samples. Such an assay is likely to be based on real-time PCR technology as this has several advantages over competitive PCR. In addition, primers could be designed to detect other AGs and subgroups. A test distinguishing between the other IGS1 types of AG2-1 would be useful since these differ in virulence (Chapter 4). Primers for these subgroups could be designed from the IGS region of rDNA, elongation factors, β-tubulin and cytochrome b genes. Sequencing of the later two genes and comparison between AGs may give insights into the differences in fungicide sensitivity for pencycuron and azoxystrobin. Analysis of the β-tubulin between AGs would give also insights into how temperature affects growth rate, as the β-tubulin gene is known to be involved in influencing the optimum growth temperature in yeast (Richards *et al.*, 2000). In fact it may be possible for the design of an assay to detect such sequences and offer a prediction of fungicide and temperature sensitivity.

In this work, an assay that can detect AG3PT in plant and soil has been developed. The assay exhibited no cross-reaction with DNA from other potato pathogens or other AGs. Such an assay maybe useful for studies into

diseases caused by *R. solani*, such as screening large numbers of isolates for AG3PT. The assay has the potential for use in diagnosis and monitoring the *Rhizoctonia* diseases in growing crops, but is limited in that it cannot currently successfully quantify the amount of AG3PT in soil.

7. Characterisation of *Rhizoctonia solani* isolates

7.1 Introduction

7.1.1 Characterisation of *Rhizoctonia solani* isolates

Several methods, including fatty acid analysis, DNA analysis, hyphal fusion compatibility and determination of thiamine requirement (Kuninaga, 2002), have been used to characterise isolates of *Rhizoctonia solani.* Such tests have been vital for the discovery of subgroups within AGs (Table 7.1), and the continued phenotypic and genotypic characterisation of *R. solani* may reveal useful markers with which to study populations in the future. This chapter describes several approaches for the characterisation of the collection of isolates of *Rhizoctonia* assembled for this study (Table 2.1).

Methods of characterisation used in this study included: a determination of thiamine requirement; a determination of optimum growth temperature; *in vitro* fungicide sensitivity and the production of sclerotia *in vitro*.

Table 7.1 Anastomosis groups and subgroups of *Rhizoctonia solani* with details of thiamine requirement, pectic zymogram group and methods of subgrouping (adapted and updated from Kuninaga, 2002)

AG	Sub-group	Methods employed to define subgroup[1]							Zymogram group[2] (where known)	Thiamine requirement (where known)
1	1A	C	P	D	FA	ITS				Autotrophic
	1B	C	P	D	FA	ITS				Autotrophic
	1C	C	P	D	FA	ITS				Autotrophic
	1D	C	P		FA	ITS				Autotrophic
2	1	C	P	D	FA	ITS	HF	PCR	5,6	Autotrophic
	2 IIIB	C	P	D	FA	ITS	HF	PCR	4,10	Auxotrophic
	2 IV	C	P	D	FA	ITS	HF	PCR	4,10	Auxotrophic
	2-2V	C	P			ITS				
	2 LP	C	P		FA	ITS	HF	PCR		
	3	C	P		FA	ITS	HF	PCR		Auxotrophic
	4					ITS	HF	PCR		
	BI	C		D		ITS	HF	PCR		Auxotrophic
3	PT	C	P	D	FA	ITS			7	Autotrophic
	TB	C	P	D	FA	ITS				Autotrophic
4	HG-I	C		D	FA	ITS			8	Autotrophic
	HG-II	C		D	FA	ITS				Autotrophic
	HG-III	C			FA	ITS				Autotrophic
5										Auxotrophic
6	HG-I	C				ITS				Autotrophic
	GV	C				ITS				Autotrophic
7										Autotrophic
8									1-1 to 1-5	Autotrophic
9	TP			D						Autotrophic
	TX			D						Autotrophic
10									9	Auxotrophic
11									3	Auxotrophic
12										
13										

[1]C, cultural type, P; pathogenicty; D, DNA-DNA hybridisation; FA, Fatty acid analysis; ITS, rDNA ITS sequence and/or RFLP analysis; HF, Hyphal fusion; PCR, PCR analysis.
[2]Pectic zymogram pattern group (MacNish and Sweetingham, 1993; MacNish *et al.*, 1994).

7.1.2 Thiamine requirement amongst isolates of *Rhizoctonia solani*

Several studies, particularly those dealing with recently discovered anastomosis groups or subgroups, have characterised isolates according to thiamine requirement (Carling *et al.*, 1987; Carling *et al.*, 1994; Carling *et al.*, 2002a; MacNish *et al.*, 1994; MacNish *et al.*, 1995). Thiamine (vitamin B1) is an essential co-enzyme in cellular respiration and several AGs and subgroups

need an exogenous thiamine supply, as they cannot synthesise the vitamin. Determination of thiamine requirement may therefore help in AG identification by eliminating certain groups. For example, isolates belonging to AG2-2, AG5 and AG11 are all known to be auxotrophic for thiamine (Table 7.1). Determination of thiamine requirement is also essential for identification of AG9 subgroupings, where isolates can be divided into AG9TX or AG9TP on the basis of thiamine auxotrophy or autotrophy respectively (Carling *et al.,* 1987). In this study, the thiamine requirement of isolates obtained in Chapter 3 was determined to see if differences existed between this study and previously published work. It was also important to determine whether thiamine requirement was consistent within all the new IGS1 subgroupings of AG2-1 described previously in this study and also if the thiamine requirements of the UK AG5 isolates in this study are consistent with overseas AG5 isolates described in other studies.

7.1.3 Differences in optimum growth temperatures amongst isolates of *Rhizoctonia solani*

Temperature can have a significant effect on disease initiation and development: Carling and Leiner (1990) showed that *R. solani* AG3 killed more potato sprouts at 10°C that at 15.5 or 21.1°C, whilst AG5 only caused damage to sprouts at 15.5 and 21.1°C. Such differences, which may be due to differences in optimum growth temperature between AG groups, have also been noted in AG3 and AG4. Anguiz and Martin (1989) determined the optimum growth temperature for potato AG3 and AG4 isolates to be between

20-25°C and 25-28°C respectively and suggested that these results could explain the distribution of the isolates in their study: infection by AG3 was found to be common on potato crops growing in cooler highland areas whilst AG4 frequently initiated disease in warmer lowland areas in Peru.

Temperature has also been determined to be an important factor influencing rhizoctonia disease of bulb crops. Doornik (1981) was able to differentiate *R. solani* isolates originating from tulip, iris and lettuce into 'warm-preferring' and 'cold-preferring' types. All these isolates had an optimum growth temperature of 25°C but the 'cold-preferring' isolates were able to grow on PDA plates incubated at 5°C whilst the 'warm-preferring' isolates did not grow at this temperature. At temperatures greater than 13°C, bulb crops were mainly infected by the 'warm-preferring' isolates, whilst at temperatures below 13°C the 'cold-preferring' isolates predominated. Therefore in addition to optimum growth temperature, the ability of the isolate to grow at specific temperatures may be an important consideration. In this study the growth rate of isolates in the culture collection was determined at a range of temperatures and the optimum growth temperature determined.

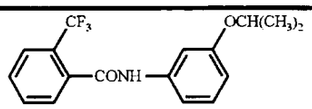
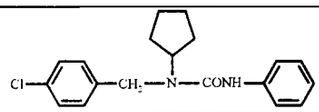
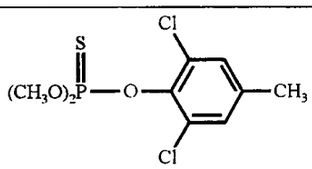
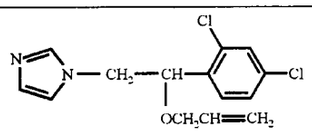
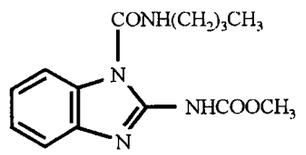
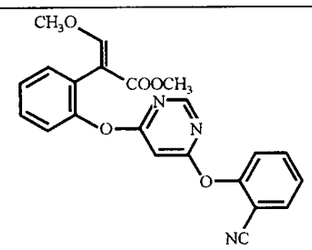
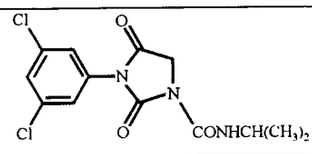
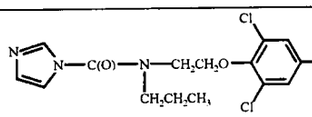
7.1.4 Sensitivity of isolates of *Rhizoctonia solani* to fungicides

The sensitivity of *R. solani* isolates to several fungicides (Carling *et al.*, 1990; Kataria *et al.*, 1991a; Kataria *at al.*, 1991b; Sumner, 1987; Campion *et al.*, 2003) and also the antibiotic gliotoxin (Jones and Pettit, 1987) is known to differ. Such differences can be explained by the development of resistance due

to selection pressure, as in the case of tolclofos-methyl resistance (van Bruggen and Arneson, 1984). Alternatively, isolates can vary in their reaction due to a natural insensitivity, and several fungicides are limited in their activity against members of a particular AG. Carboxin, furmecyclox, triadimenol, propiconazole and the pyrimidine fungicides fenarimol and nuarimol differ in their ability to control various AGs of *R. solani* (Kataria and Gisi, 1999). However, the phenylurea fungicide pencycuron has shown the highest level of selectivity between AGs with strong activity against AG1, AG2-1, AG2-2, AG3, AG6 and AG9 and little or no activity against AG5, AG7 and AG8. Some isolates of AG4 have been reported to be highly sensitive to pencycuron whilst others are insensitive. The existence of such fungicide selectivity has led to the speculation that their continued use may force a change in the pathogen population towards insensitive AGs.

This study determined the *in vitro* sensitivity of isolates in the *R. solani* culture collection to several fungicides in an attempt to discover evidence of selectivity between groups and resistance amongst isolates. Fungicides were chosen on the basis of availability, existing evidence of selectivity and relevance to rhizoctonia potato disease. The fungicides tested in this study, the chemical structure, class and mode of action are listed in Table 7.2. This study also determined the baseline sensitivity values for the fungicides flutolanil and azoxystrobin. Prior to this study, these chemicals were not used to control rhizoctonia potato disease in the UK.

Table 7. 2 Structure, active ingredient, class and mode of action of fungicides tested in this study

Active ingredient	Structure	Proprietary name	Fungicide class	Mode of action
Flutolanil		RhiNo	Carboxamide	-Inhibition of succinate oxidation[1]
Pencycuron		Monceren	Phenylurea	-Microtubule formation and/or function[2]
Tolclofos-methyl		Rizolex	Aromatic hydrocarbon	-Lipid peroxidation -Cytokinesis inhibition -Interference of phosphatidylcholine biosynthesis[3]
Imazalil		Fungazil	Imidazole	Demethylation inhibitor[3]
Benomyl		Benomyl	Benzimidazole	-Antimicrotubular action -Inhibition of mitochondrial respiration[3]
Azoxystrobin		Amistar	Strobilurin	Cytochrome b/c complex III site[4]
Iprodione		Rovral	Dicarboximide	-Unknown but similar to aromatic hydrocarbon. Cross resistance with tolclofos methyl[4]
Prochloraz		Prelude	Imidazole	Demethylation inhibitor[3]

[1]Hirooka *et al.* (1990); [2]Leroux *et al.* (1990); [3]Kataria and Gisi (1999); [4]Uesugi (1998)

7.1.5 Ability of *Rhizoctonia solani* isolates to produce sclerotia *in vitro*

The ability to produce sclerotia on potato tubers has been observed to differ greatly between AGs in field situations, as discussed in Chapter 4. However,

production of sclerotia under field conditions is likely to be influenced by volatile tuber exudates (Dijst, 1988) and certain AGs, particularly AG3, may be more receptive to some potato exudates than others. Therefore, an experiment was conducted to determine whether the other anastomosis groups associated with potato also had the ability to produce sclerotia *in vitro*. Sclerotial production has been shown to differ between AGs in previous *in vitro* studies. Naiki and Ui (1978) found that isolates of AG2-1 produced sclerotia abundantly on agar compared to isolates belonging to AG1, AG2-2, AG3, AG4 and AG5. They also found that one isolate of AG3 did not form any sclerotia. Anguiz and Martin (1989) compared the growth of AG3 and AG4 on PDA and found that whilst AG3 isolates all produced large numbers of sclerotia, AG4 isolates only produced a few sclerotia. Harikrishnan and Yang (2004) compared sclerotial production on PDA and observed that AG1 produced the greatest number of sclerotia compared to representative isolates of AG2-2, AG4 and AG5. Hyakumachi and Ui (1987) also found differences within an AG: isolates of AG2-2 that did not have the abilty to self-anastomose did not produce any sclerotia on PDA, whereas isolates that did self-anastomose produced sclerotia abundantly.

7.16 Aim of the study

The aim of this study was to characterise isolates of *R. solani* in their sensitivity to fungicides, growth response to temperature, *in vitro* sclerotia production and determine the presence of thiamine auxotrophisms. This characterisation will provide an insight into the diversity and differences of

isolates within and between AGs, enabling conclusions about the structure of the population of *R. solani* in potatoes.

7.2 Materials and Methods

7.2.1 Determination of thiamine requirement

Plates of 1.5% Czapek Dox Agar (CDA) were prepared with modified Czapek Dox liquid media (Oxoid, Basingstoke, UK) and Agar No.2 and inoculated with the appropriate isolate and incubated at 25°C. Once the CDA plates were completely colonised, 5 mm plugs were removed and were used to inoculate 100 ml conical flasks containing 30 ml Czapex Dox broth amended with 1 ml thiamine solution to a final concentration of 10^{-5}M thiamine hydrochloride (MacNish *et al.,* 1994) or 1 ml SDW. Each treatment was replicated three times.

After two weeks growth at 25°C, mycelia were removed by filtering the contents of the flask through lens tissue. Mycelia were subsequently washed with distilled water and dried overnight and weighed. Thiamine auxotrophy for each isolate was determined by calculating if thiamine treated mycelia were significantly greater in weight than untreated mycelia (t-test assuming unequal variances, $P<0.05$).

7.2.2 Determination of growth rate and optimum growth temperature

Isolates of *R. solani* were taken from long-term storage (Chapter 2) and incubated on PDA plates at 25°C. Once the plates were fully colonised, 7 mm plugs of each isolate were removed using a cork borer, and placed in the

centre of 3-cam 92 mm Petri dishes containing PDA. Cultures were incubated at temperatures ranging from 10°C to 37°C. Each isolate-temperature combination was replicated three times; Petri-dishes were assorted randomly, placed in plastic bags and incubated for 45 hours. Data loggers (Tiny Tag Plus, Gemini Data Loggers UK Ltd, Chichester) were placed in each incubator and the temperature was recorded every 10 minutes to determine an accurate mean temperature. After incubation, colony diameter was recorded (mm) with measurements along two perpendicular axes. Colonies were also checked for purity of culture.

7.2.3 Sensitivity of *Rhizoctonia solani* isolates to fungicides

7.2.3.1 Fungicide samples and preparation of media

Fungicide samples (Table 7.2) were supplied by the relevant chemical company and stored at room temperature. Each fungicide was diluted to a 6250 mg l^{-1} stock solution. Dilutions of all fungicides except benomyl were made in sterile distilled water (SDW). Benomyl dilutions were made in dimethyl sulphoxide (DMSO)(Sigma) due to the insolubility of benomyl in water. Stock solutions were stored at room temperature in sterile polypropylene 50-ml tubes (Sarstedt, Leicester, UK).

Assays were based on the poisoned food technique (Dhingra and Sinclair, 1995). The growth media used for the assay was Potato Dextrose Agar

(LabM). After autoclaving the PDA in 1000-ml Duran flasks, media were allowed to equilibrate to 45°C for one hour before amending with the appropriate fungicide solution. Dilutions and amendments were all done in sterile 50-ml polypropylene tubes

7.2.3.2 Citing test

To determine the concentration of fungicide to be used for the main test, a citing test was performed with isolates representative of each anastomosis group or subgrouping if appropriate. Concentrations used for the citing test ranged from 0.001 to 50 mg l^{-1}. No replication was used in the citing tests. Based on interpretations of the results of the citing test, the appropriate concentrations were decided for use in the main test.

7.2.3.3 Main test

Stock fungicide solutions were diluted to the required concentrations and 10 ml of the diluted stock was added to 990 ml of media in a laboratory fume cupboard to achieve the desired end concentration. For control (unamended plates), 10 ml of SDW (or DMSO where appropriate) was added instead of fungicide. The amended media was thoroughly mixed and poured into sterile 3-cam 92 mm Petri dishes (Sarstedt). Inoculum consisted of a 7 mm fully colonised PDA plug of the appropriate isolate. Plugs were taken from the appropriate plate using a flamed cork borer and transferred to the amended

media plates using a flamed scalpel. Each isolate-fungicide combination was replicated three times.

Following inoculation, plates were labelled, randomised, put into plastic bags and placed into an incubator set at 25±2°C. Plates were incubated until the control plates were within 1 cm of the edge of the plate (2-4 days, depending on the isolate). After incubation, colony diameter was recorded (mm) with measurements along two perpendicular axes. Colonies were also checked both macroscopically and microscopically for purity of culture.

7.2.3.4 Analysis of data

For each isolate-fungicide concentration combination, the percentage inhibition of growth as compared to the control was determined using the formula in equation 7.1.

Equation 7.1

% Inhibition = 100 - ((T/C) x 100))

Where T = treatment mean measurement and C = Control mean measurement.

The average percentage inhibition was then plotted against the logarithm of the fungicide concentration and a linear regression equation determined. The effective concentration for 50% fungal growth inhibition value (EC_{50}) was then calculated from the linear regression equation.

7.2.4 Ability of *Rhizoctonia solani* isolates to produce sclerotia *in vitro*

Rhizoctonia isolates were taken from long-term storage and grown on PDA at 25°C until the plate was fully colonised. Plates were checked for purity and plugs of 7 mm diameter were taken for each isolate and placed in the centre of 92 mm Petri dishes of PDA. Each plate was sealed with parafilm, replicated three times and incubated at 25±2°C for 18 days. Sclerotial coverage was then measured using a transparent grid with subdivisions of 5 mm^2. Subdivisions containing a sclerotial area of 50% or more were scored and percentage sclerotia coverage calculated.

7.3 Results

7.3.1 Determination of thiamine requirement

Mean mycelial weights of isolates of various AGs grown in thiamine amended and unamended media, are given in Table 7.3 along with the deduced ratio of thiamine:non-thiamine growth, and a determination of the thiamine requirement category.

Table 7. 3 Mean mycelial weight of isolates grown in Czapek Dox broth with and without thiamine and determined thiamine requirement

AG	Isolate	Mycelial weight (g)		Ratio (+/-)	Thiamine Requirement
		With Thiamine	Without thiamine		
AG2-1	R22	0.48	0.38	1.26	Autotrophic
	R42	0.30	0.21	1.43	Autotrophic
	X52	0.48	0.42	1.14	Autotrophic
	X81	0.06	0.08	0.75	Autotrophic
	Y2	0.07	0.11	0.64	Autotrophic
	Y3	0.17	0.13	1.31	Autotrophic
	Y25	0.13	0.17	0.76	Autotrophic
	Y63	0.14	0.27	0.52	Autotrophic
	Z1	0.11	0.11	1.00	Autotrophic
AG2-2 IIIB	R70	0.18	0.07	2.57	Auxotrophic
AG2-2 IV	R73	0.22	0.07	3.14	Auxotrophic
	R75	0.29	0.08	3.63	Auxotrophic
AG2-3	R78	0.19	0.09	2.11	Auxotrophic
AG3PT	UN	0.57	0.58	0.98	Autotrophic
	X40	0.78	0.64	1.22	Autotrophic
	Y29	0.15	0.15	1.00	Autotrophic
AG4	R87	0.17	0.15	1.13	Autotrophic
	R89	0.22	0.32	0.69	Autotrophic
AG5	R48	0.11	0.07	1.57	Auxotrophic
	R52	0.13	0.06	2.17	Auxotrophic
	T1	0.14	0.04	3.50	Auxotrophic
	Y55	0.15	0.05	3.00	Auxotrophic
AG8	R28	0.12	0.12	1.00	Autotrophic

Mycelial weights of isolates grown in unamended media were significantly lower than mycelial weights from thiamine amended media ($P<0.05$) for isolates belonging to AG2-2, AG2-3 and AG5 and were consequently judged

to be thiamine autotrophic. No significant differences in the mycelial weight of isolates from AG2-1, 3PT, 4 and 8 grown in thiamine amended or unamended media were observed, and these isolates were determined to be thiamine autotrophic. The ratio of mycelial weights from media without thiamine to that from media with thiamine ranged from 0.52 to 1.43 for autotrophic isolates and from 1.57 to 3.62 for the auxotrophic isolates. MacNish *et al.,* (1994) defined isolates as autotrophic for thiamine if the ratio was 1.5 or less.

7.3.2 Growth rate and optimum growth temperature of *Rhizoctonia solani* isolates

Hyphal growth rate at each temperature tested is given in Table 7.4. Temperatures given are the average data logger reading over 45 h. Temperatures at which the highest growth rates were recorded were between 20.5 and 28.9°C. Three isolates of AG2-1 (R42, X46 and Y2) and one isolate of AG3PT (X40) recorded their highest growth rate at the relatively low temperature of 20.5°C. Eight isolates recorded their highest growth temperature at 28.9°C including the AG1-IC isolate, R62, three AG2-2 isolates (R38, R68, and R73), one isolate each of AG4 (R86) and AG5 (R48) and the AG7 isolate, R97. The AG1-IC isolate, two of the AG2-2 isolates and the AG7 isolate all grew better at high temperatures (31.6°C and 33.9°C) compared to other isolates. Isolates of AG4 grew poorly at 7.8°C, and no growth at all was recorded at this temperature for isolate R86, however R86 did grow better than the other AG4 isolates at higher temperatures.

Table 7. 4 Growth rate (mm day^{-1}) and optimum growth temperature of *Rhizoctonia solani* isolates from the culture collection

Isolate	AG	Temperature (°C)						
		7.8	13.9	20.5	25.7	28.9	31.6	33.9
R59	1-IA	0.0	4.2	17.5	30.6*	25.0	4.1	4.1
R60	1-IB	4.6	15.2	22.1	34.2*	31.7	2.2	2.2
R61	1-IB	1.4	8.2	22.1	35.9*	31.2	1.2	1.2
R62	1-IC	3.5	13.3	25.3	35.0	40.9*	nt	14.1
R22	2-1 l	2.7	6.9	15.1	15.2*	9.0	1.1	1.1
R42	2-1 l	5.4	9.4	15.6*	15.1	8.1	0.9	0.9
X46	2-1 n	3.0	8.4	18.0*	17.9	9.2	0.0	0.0
X52	2-1 n	0.8	4.1	16.5	17.0*	8.8	0.6	0.6
Y25	2-1 n	4.8	10.2	22.1	24.7*	21.5	3.9	3.9
Y3	2-1 n	3.4	8.9	21.5	23.0*	18.0	1.5	1.5
X81	2-1 s	5.5	11.0	17.7	20.2*	13.1	1.1	1.1
Y2	2-1 s	3.8	5.6	29.3*	18.7	9.3	0.2	0.2
R38	2-2	3.3	9.2	20.9	24.6	25.1*	13.7	13.7
R68	2-2IIIB	1.3	11.4	20.8	34.0	41.7*	15.7	15.7
R71	2-2IV	1.6	10.1	18.3	24.2*	22.3	3.9	3.9
R72	2-2IV	2.2	10.5	18.8	19.0*	12.8	0.6	0.6
R73	2-2IV	0.5	8.7	19.7	24.9	25.4*	3.8	3.8
R75	2-2IV	0.8	7.6	21.4	26.3*	24.2	4.6	4.6
R78	2-3	2.7	12.0	17.5	30.6*	25.0	4.1	4.1
R98	2-BI	0.6	4.8	20.1	21.1*	14.4	0.9	0.9
I3	3PT	2.9	8.8	16.4	18.4*	13.3	1.2	1.2
UN	3PT	3.3	8.0	18.8	20.3*	13.6	0.6	0.6
X22	3PT	2.3	7.0	18.0	21.1*	11.3	0.4	0.4
X34	3PT	3.3	9.7	16.0	18.4*	7.9	1.4	1.4
X40	3PT	3.8	9.2	18.1*	11.2	8.2	0.9	0.9
R86	4	0.0	6.0	22.0	30.3*	29.9	5.0	5.0
R87	4	1.4	8.2	18.3	21.7*	21.2	1.9	1.9
R89	4	1.4	8.9	17.7	22.0*	18.8	1.6	1.6
R90	4	1.7	8.9	18.5	23.3*	21.5	3.9	3.9
R48	5	4.1	8.8	22.6	28.1	29.1*	2.1	2.1
R52	5	3.8	7.2	19.1	26.6*	26.5	4.5	4.5
T1	5	3.8	11.4	24.3	30.7*	30.4	3.0	3.0
R26	6	2.4	7.0	12.5	18.3*	17.0	2.2	2.2
R54	6	1.4	5.0	10.5	15.4*	12.8	0.5	0.5
R97	7	0.3	1.7	11.9	17.7	26.5*	24.7	24.7
R28	8	2.7	7.3	12.2	12.9*	11.3	1.0	1.0

*Temperature at which the recorded growth rate was highest; nt, not tested.

Considerable variation existed between growth rates at different temperatures in isolates of AG2-1. Three AG2-1 isolates, R42, X46 and Y2 grew well at low temperatures with growth rates above 3 mm day^{-1} at 7.8°C but not well at

temperatures over 28°C. The AG2-1 isolate, Y25, compared to the other isolates of AG2-1 and AG3 tested had good growth rates at all temperatures tested, none below 3.9 mm day^{-1}. This may explain its highly virulent nature observed in Chapter 3. Less variability in growth rates was observed in AG3, but all had a similar growth rate of around 3 mm day^{-1} at 7.8°C.

7.3.3 Sensitivity of individual *Rhizoctonia solani* isolates to fungicides

Table 7.5 shows the EC_{50} values (mg l^{-1}) for each isolate for each fungicide tested. Forty-two isolates representing eight anastomosis groups and various subgroups were tested. In addition to the fungicides listed in Table 7.5, prochloraz was tested; all isolates listed in Table 7.5 had an EC_{50} of above 50 mg l^{-1} for prochloraz.

Variations in sensitivity between isolates existed for all the fungicides listed in Table 7.5. Large variations in sensitivity existed for azoxystrobin; the two AG1-IB isolates tested (R60 and R61) and the AG7 isolate (R97) were particularly sensitive to azoxystrobin, all with an EC_{50} under 0.26 mg l^{-1}. All the remaining isolates tested had an EC_{50} for azoxystrobin of above 1.4 mg l^{-1}. Several had an EC_{50} of above 10 mg l^{-1}, the maximum concentration tested in this study.

EC_{50} for benomyl ranged from 0.41 mg l^{-1} to 4 mg l^{-1} with only the three AG1 isolates tested (R58, R60 and R62) and one isolate of AG4 (R87) having an EC_{50} under 1 mg l^{-1}. With flutolanil, all isolates except AG2-BI isolate (R98)

had an EC_{50} from 0.01 to 0.87 mg l^{-1}. The AG2-BI isolate however, was considerably out of this range with an EC_{50} of 2.48 mg l^{-1}, suggesting this subgroup may have some insensitivity to the fungicide.

Table 7. 5 EC_{50} values (mg l^{-1}) for seven fungicides tested against 42 isolates of *R. solani* representing anastomosis groups 1 to 8

Code	AG	Azoxy-strobin	Benomyl	Fluto-lanil	Imazalil	Iprodione	Pency-curon	Tolclofos methyl
R58	1-IA	9.17	0.784	0.10	1.57	0.79	0.03	0.19
R59	1-IA	4.20	nt	nt	6.56	0.44	0.04	nt
R60	1-IB	0.01	0.41	0.01	6.18	0.34	0.04	0.08
R61	1-IB	0.25	nt	nt	<1	0.44	0.02	0.11
R62	1-IC	>10	0.944	0.06	3.16	0.55	>50	0.08
R22	2-1 l	>10	2.37	0.04	5.54	0.68	0.07	0.15
R42	2-1 l	>10	2.33	0.06	7.73	1.20	0.10	0.20
X1	2-1 l	>10	3.90	0.05	21.23	1.35	0.09	0.16
X46	2-1 n	>10	3.63	0.11	16.28	0.94	0.09	0.10
X81	2-1 s	>10	4.02	0.17	10.52	0.80	0.08	0.11
Y2	2-1 s	6.10	3.13	0.15	10.96	0.75	0.07	0.09
Y3	2-1 n	>10	2.63	0.13	17.74	0.40	0.05	0.10
Y25	2-1 n	8.13	2.69	0.11	22.14	0.70	0.15	0.22
Y63	2-1 s	2.15	2.09	0.01	1.49	0.52	0.07	0.16
Z1	2-1 s	>10	2.34	0.12	8.14	0.58	0.09	0.20
R23	2-2	6.36	1.54	0.26	10.42	R	0.05	R
R38	2-2	7.64	2.66	0.34	52.55	0.60	0.08	0.23
R70	2-2IIIB	>10	5.24	0.87	3.30	R	0.09	R
R71	2-2IV	3.60	3.09	0.16	23.51	0.66	0.03	0.18
R73	2-2IV	>10	2.40	0.24	38.35	0.93	0.06	0.12
R75	2-2IV	>10	2.58	0.40	26.25	0.69	0.07	0.12
R78	2-3	3.07	1.45	0.13	5.73	1.00	>50	0.09
R98	2-BI	7.17	1.18	2.48	2.90	0.55	0.02	0.18
X34	3PT	>10	3.13	0.15	24.81	1.03	0.12	0.27
X40	3PT	9.02	1.57	0.18	24.89	0.93	0.06	0.08
Y29	3PT	>10	2.13	0.14	33.89	1.42	0.07	0.13
UN	3PT	>10	2.64	0.19	29.54	0.87	0.06	0.09
I3	3PT	>10	2.44	0.09	8.07	0.47	0.06	0.16
R51	4	>10	1.30	0.07	<1	1.10	0.14	0.12
R86	4	>10	1.31	0.14	6.03	0.79	0.07	0.14
R87	4	>10	0.62	0.05	34.33	0.62	>50	0.20
R89	4	>10	1.27	0.08	17.71	0.49	>50	0.18
R90	4	>10	1.23	0.09	22.10	0.77	>50	0.06
R48	5	>10	1.61	0.07	10.00	0.70	>50	0.10
R52	5	9.98	2.24	0.17	17.26	1.06	>50	0.19
T1	5	1.44	2.48	0.09	13.68	0.70	>50	0.10
Y55	5	3.71	1.97	0.07	12.93	0.56	>50	0.10
R26	6	8.42	1.21	0.05	8.92	0.76	0.12	0.08
R54	6	>10	1.96	0.11	49.29	2.00	0.19	0.11
R97	7	0.05	2.54	0.23	<1	0.62	>50	0.13
R28	8	>10	1.54	0.17	7.62	0.60	>50	0.68

R, possible fungicide resistance present in that isolate; nt, fungicide-isolate combination not tested.

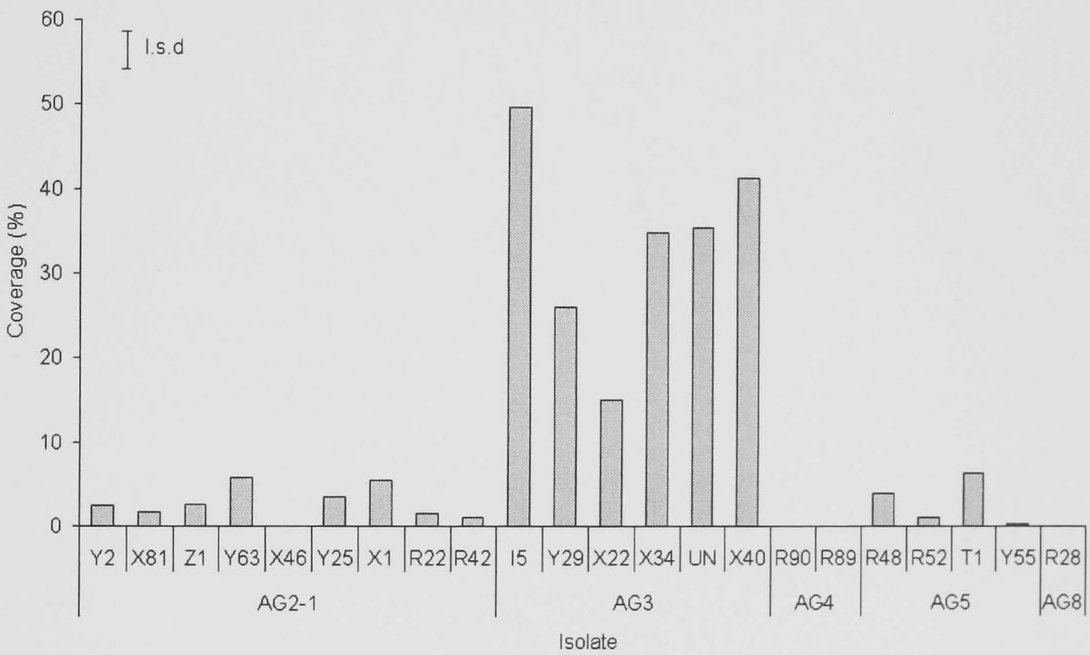
Isolates were less sensitive to imazalil than all other fungicides except prochloraz. Only four isolates (R61, R68, R51 and R97) tested in this study had an EC_{50} for imazalil less than 1 mg l^{-1} and these were from four different AGs (AG1-IB, AG2-2IIIB, AG4 and AG7), suggesting that sensitivity to the fungicide is not linked to AG in this instance.

EC_{50} for iprodione ranged from 0.28 to 2 mg l^{-1} except in isolates R23 and R70. EC_{50} values for tolclofos-methyl ranged from 0.08 to 0.68 mg l^{-1} except for isolates R23 and R70 again. Colony diameter actually increased in R23 and R70 when the isolates were exposed to increasing concentrations of iprodione or tolclofos-methyl possibly indicating the presence of fungicide resistance.

Pencycuron resulted in the most variation for fungicidal sensitivity with isolates either being highly sensitive (EC_{50} < 0.16 mg l^{-1}) or insensitive (EC_{50} > 50 mg l^{-1}). All isolates of AG1-IA, AG1-IB, AG2-1, AG3PT and AG6 were sensitive to pencycuron whereas isolates of AG1-IC, AG2-3, AG5 and AG8 were insensitive. Variability within an AG occurred within AG2-2 and AG4, all isolates of AG2-2 but two were sensitive to pencycuron and all but two of the five AG4 isolates were insensitive to pencycuron.

7.3.4 Ability of *Rhizoctonia solani* isolates to produce sclerotia *in vitro*

Differences were observed in the number of sclerotia produced *in vitro* between AG3PT and the other groups tested (Figure 7.1). AG3PT produced most sclerotia with between 15 to 50% of the plates covered with sclerotia. Isolate X22 produced the least with only 15% coverage, however the coverage was still significantly greater than that of any non-AG3 isolate. Isolate I5 produced the most sclerotia of any isolate tested with almost 50% of the culture area displaying sclerotia.



l.s.d = 5.35, s.e.d. = 2.69, cv% = 38.9

Figure 7.1 Percentage coverage of sclerotia on plates of PDA for isolates of *R. solani* of various AGs after 18 days incubation at 25°C

All isolates of AG2-1 produced less than 6% coverage by sclerotia, with one isolate, X46, generating no sclerotia. No statistical differences for sclerotial coverage were observed between the different IGS1 types of AG2-1. Of the AG5 isolates, T1 produced the most sclerotia (6.5% coverage), but this was

only significantly more than the Y55 isolate, which produced the least sclerotia on average. No sclerotia were observed in the two isolates of AG4 (R89 and R90) and the AG8 isolate (R28).

7.4 Discussion

In this study the experiments conducted to characterise isolates of *R. solani* of various AGs have shown that considerable differences exist between AGs. Differences in thiamine, optimum growth temperature, sensitivity to fungicides and sclerotia production was often evident between AGs.

7.4.1 Thiamine requirement of *Rhizoctonia solani* isolates

Thiamine requirement differed between AGs as indicated in previous work (Kuninaga, 2002). Isolates from all IGS1 types described previously of AG2-1 were autotrophic for thiamine indicating this trait is conserved across AG2-1. This is concordant with other studies, Steven-Johnk and Jones (1993) and MacNish *et al.* (1994) both found that isolates of AG2-1 do not require thiamine. This trait is not conserved across AG2, as AG2-2 and AG2-3 isolates both in this study and in previous studies with AG2-BI (Kuninaga, 2002) were not autotrophic for thiamine. All isolates of AG5 were auxotrophic, which is consistent with other studies (Fenille *et al.*, 2002; Meyer *et al.* 1998; Nicoletti *et al.*, 1999; MacNish *et al.*, 1994). The significance of thiamine requirements in the epidemiology and ecology of the pathogen is unknown to date.

7.4.2 Hyphal growth rate and optimum growth temperature *in vitro*

The growth rate of isolates on plates differed between AGs and sometimes within AGs. Highest growth rates were recorded at 20.5°C or 25.7°C for isolates from Great Britain as opposed to 28.9°C that was observed for some isolates from warmer climates, this does suggest some adaptation to habitat. Differential growth rates between AG1, AG2-2, AG4 and AG5 in response to temperature were also observed by Harikrishnan and Yang (2004). In their study, isolates from warmer climates exhibited a low growth rate at lower temperatures; which affected their survival ability at lower temperatures.

Carling and Leiner (1990) observed that temperature could influence disease development, with AG5 only causing severe infection of potato stems at temperatures of 15.5 and 20°C as opposed to AG3, which could cause severe infection at 10°C as well. This is not reflected in the *in vitro* growth as AG5 grows as well, if not better than AG3 isolates at all temperatures, this suggests that virulence is regulated by more complex mechanisms in AG5. However *in vitro* growth rates may help to explain why one isolate of AG2-1 (Y25) was highly virulent nature to potato. Y25 exhibited good growth at all temperatures. Previously in AG2-1 *in vitro* growth rates at different temperatures have been related to virulence (Doornik, 1981). Growth rates also suggested that AG2-1 is a diverse group, whilst AG3 is homogenous.

7.4.3 Sensitivity of *Rhizoctonia solani* isolates to fungicides

Isolates show differences in their sensitivity to the fungicides with diverse chemical structures. Most fungicides tested here had activity against most isolates representing AGs 1 to 8, except prochloraz and imazalil. Previous studies have stated that prochloraz has poor activity against *R. solani* (Carling *et al.,* 1990; Smiley *et al.,* 1990; Kataria *et al.,* 1991a; Kataria *et al.,* 1991b). Poor activity has been observed previously with imazalil (Kataria and Gisi, 1989), however in this study all isolates had an EC_{50} less than 50 mg l^{-1}, some isolates having an EC_{50} less than 1 mg l^{-1} suggesting that imazalil does possess some fungitoxic ability, unlike prochloraz, also in the imidazole class of fungicides, which had an EC_{50} at least above 50 mg l^{-1} for all isolates tested.

In this study EC_{50} values were low for iprodione and flutolanil, values were similar to those observed by Campion *et al.* (2003) for AG2-1, AG3 and AG5. Flutolanil showed good inhibition of all isolates tested except the AG2-BI isolate which had a considerably higher EC_{50} than the other isolates, this may suggest that this isolate or the whole AG2-BI subgroup could possess some degree of insensitivity; more isolates would need to be tested to determine this. EC_{50} values could not be determined for two isolates of AG2-2 for iprodione and tolclofos-methyl. In these instances, hyphal growth increased with increasing concentrations of fungicide suggesting that these isolates possess resistance to these fungicides. Resistance has been found in *R. solani* isolates to tolclofos-methyl (van Bruggen and Arneson, 1984) and iprodione resistance has been found in turf grass isolates of binucleate *Rhizoctonia*

(Sanders *et al.,* 1978). The resistance of AG2-2 isolates to both iprodione and tolclofos-methyl is likely to be an example of cross-resistance, which is believed to occur, despite structural differences between iprodione and tolclofos-methyl, as these fungicides share a similar mode of action (Kataria and Gisi, 1999). For all other isolates EC_{50} values were low for tolclofos-methyl, concordant with previous studies (as reviewed in Kataria and Gisi, 1996).

Sensitivities to benomyl were similar to previous work (Carling *et al.,* 1990) ranging from 0.4 to 4 mg l^{-1}; isolates of AG1 tested seemed to have a higher sensitivity to benomyl, which may possibly be a genetic trait of that group. Azoxystrobin exhibited a wider range of sensitivities, some isolates, particularly the AG1-IB isolates and the AG7 isolate were extremely sensitive to the fungicide (EC_{50}<0.25 mg l^{-1}) whilst some had an EC_{50} greater than 10 mg l^{-1}. However azoxystrobin does provide good control of *R. solani* in the field (Hilton *et al.,* 2004; Wale *et al.,* 2004). With azoxystrobin at 10 mg l^{-1}, a decrease in colony radius was observed for isolates but not enough to determine an accurate EC_{50} value, suggesting that azoxystrobin may have a fungistatic as opposed to a fungicidal activity towards some AGs, or through some interaction with the plant provide control against the disease. This is similar to the fungicide fosetyl-al, where little or no fungitoxic ability is observed *in vitro* but good efficacy is observed in the field. This may be due to a higher *in vivo* fungitoxic activity or due to induced plant resistance (Useugi, 1998).

Many isolates were highly sensitive to pencycuron, including all isolates of AG1-IA, AG1-IB, AG2-1 and AG3 and AG6. However, several isolates exhibited insensitivity to this fungicide; AG5, AG7 and AG8 have been observed to be insensitive to pencycuron previously (Kataria *et al.,* 1991a; Kataria *et al.,* 1991b; Olaya *et al.,* 1994). In this study isolates of AG1-IC and AG2-3 were observed to be insensitive to pencycuron, which has not been observed before.

Previous studies have stated that isolates of AG4 can be either sensitive or insensitive to pencycuron (Kim *et al.,* 1996). AG4 is known to be a diverse group and such insensitivity may correspond to the previously determined subgroups (Kim and Yamaguchi, 1996). In this study, isolates R86 was sensitive to pencycuron and hyphal growth rates at different temperatures indicated a preference for growing at higher temperatures. This is similar to the results obtained by Kim and Yamaguchi (1996), who observed that sensitivity to pencycuron was negatively correlated with poor hyphal growth at 30°C. Subsequently, some of the isolates used by Kim *et al.* (1996) have been assigned to the subgroups of AG4 HGI and HG-II (Kuninaga *et al.*, 1997). One pencycuron sensitive isolate belonged to HGI and two insensitive isolates belonged to HG-II, the pencycuron and temperature response traits may therefore be indicative of those subgroups.

Kim and Yamaguchi (1996) suggested that the differences in sensitivities may be due to membrane compositions. Through radiolabelling they observed that pencycuron bound to the membrane of a sensitive isolate of AG4 three times

as much as an insensitive isolate. The binding of pencycuron caused a decrease in membrane fluidity. The fluctuation in sensitivities may be explained by differences in membrane compositions and hence membrane fluidity (Kim and Yamaguchi, 1996). However, other studies suggest cytoskeletal microtubules are targeted by pencycuron (Ueyama *et al.,* 1990; Leroux *et al.,* 1990). It is likely that any effect on any cytoskeleton microtubules will effect membrane composition and vice versa (Kim *et al.,* 1996).

Microtubules are made up of the heterodimers of α and β-tubulin (Richards *et al.,* 2000). Changes in the β-tubulin are known to confer resitance to benomyl and are also associated with growth response to temperature (Richards *et al.,* 2000). In this study, and work by Kim and Yamaguchi (1996), insensitivity to pencycuron was linked to temperature response; this could suggest that the differences in the β-tubulin may ultimately explain sensitivity or insensitivity to pencycuron. Sequencing of the β-tubulin gene may find which regions of DNA are different and may elucidate the mechanisms of pencycuron insensitivity and hyphal growth at different temperatures.

Whilst assays determining the EC_{50} are useful for determining the baseline sensitivity of isolates to specific fungicides, detecting the development of resistance and as an initial test to see if a fungicide has activity against a particular pathogen, they cannot truly indicate the potential of the fungicide to successfully control the disease. These tests measure hyphal growth rate and not the ability of the fungicide to inactivate sclerotia on a potato tuber or

suppress infection cushion formation, therefore the test should be used in conjunction with *in vivo* studies.

7.4.4 Ability of *Rhizoctonia solani* isolates to produce sclerotia *in vitro*

AG3PT isolates produced much more sclerotia than isolates of AG2-1, AG4, AG5 and AG8. AG3 has been observed to have an ability to produce large amounts of sclerotia *in vitro* compared to AG4 previously (Anguiz and Martin, 1989). This suggests that AG3PT isolates are prolific producers of sclerotia, however this is in the absence of the host plant. The other AGs tested may be prolific sclerotia producers under different conditions. Previous work has indicated that certain herbicides can increase sclerotia production (Harikrishnan and Yang, 2001). Phosphate and magnesium have been shown increase the number and weight of sclerotia (Sumner, 1996) and cyclic adenosine monophosphate (c-AMP) can even induce sclerotia production in isolates which were previously thought to be non-sclerotial (Sharada *et al.*, 1992). Conclusions about sclerotia production ability *in vitro* should be supplementary to field work. Nevertheless this experiment exemplifies a biological difference between AG3 and the other AGs tested.

8. General discussion

8.1 Discussion

This study showed that several AGs of *R. solani* are present in potato crops in Britain. AG3PT was the predominant group; however AG2-1 and AG5 were also present. Glasshouse and field experiment data has exhibited differences in disease severity and development between AGs. The lack of relationship between stem canker and black scurf in some crops can therefore be explained by the presence of several AGs in British crops. AG3PT, the predominant AG caused the typical rhizoctonia potato disease symptoms and yield losses up to 33% by weight, similar to losses observed by Banville (1989). The other AGs found on potato crops in this study, AG5 and AG2-1 were capable of causing stem and stolon canker and yield losses of approximately 15% but were not capable of causing significant amounts of black scurf. Another anastomosis group, AG8 was not detected in potato crops, however AG8 was shown to cause severe root infections but not stem and stolon canker in field and glasshouse experiments.

Previously the importance of AG8 root infection was unknown, as AG8 infection of potato was only previously studied in a controlled environment (Hide and Firmager, 1990) and did not determine tuber yield losses. In this study, infection of AG8 was shown to reduce yield by almost 25% in two field experiments. AG3PT was capable of causing root cankers, but not as severe as those caused by AG8. Previously, the stem and stolon cankers were often

thought to be of major importance in determining yield loss, this study suggests that the root infection could be a key component determining yield loss. AG3PT however also caused high incidences of the tuber blemish disease, black scurf, and this causes an additional economic loss through a loss of quality.

Both sclerotia production *in vitro* and in field experiments were low in AG2-1, 5 and 8. This suggests that spread via seed-borne sclerotia is limited, and therefore soil inoculum may be of primary importance with these groups. In this study AG5 was isolated from couch grass stem bases found amongst an AG3PT potato stem suggesting that AG3PT predominates in potato infections. The predominance of AG3PT in mixed inoculations of potato plant with AG3 and AG5 has been observed previously (Stack *et al.,* 1999). However, a possible explanation in this instance is that the AG3PT potato infection originated from seed-borne inoculum in close proximity to the emerging potato stem, conversely the AG5 may have been present in the soil. The ability of AG3PT to disperse via seed-borne inoculum may explain its predominance in British potato crops. Long distance dispersal of AG3PT isolates via seed-borne inoculum has been observed in North America (Ceresini *et al.,* 2003).

Soil-borne inoculum and presence on other hosts may be important in AG2-1. Isolates of AG2-1 had at least the same or higher *in vitro* hyphal growth rate at all temperature compared to AG3PT isolates. Isolates of AG2-1 are capable of causing stem infection more severe than AG3PT as indicated in the glasshouse

and pathogenicity tests. In pathogenicity tests with 38 isolates representing isolates of AG2-1, AG3PT, AG5 and AG8, one AG2-1 isolate (Y25) caused the most severe stem infections. Hyphal growth rate *in vitro* of isolate Y25 was higher than for other isolates tested; this may explain the high virulence of Y25. Previously, hyphal growth rates were indicated to be an important factor in the saprophytic survival ability of isolates of *R. solani* (Harikrishnan and Yang, 2004). Enhanced hyphal growth may explain why isolates of AG2-1 are able to survive as soil-borne inoculum. Naiki (1986) reported that the sclerotia of AG2-1 are also more susceptible to invasion by *Trichoderma* species, compared AGs 1, 3, 4 and 5. This observation may suggest that AG2-1 is less adapted in the ability to cause seed-borne infection.

In this study molecular diagnostics aided the identification of AG, allowing all isolates to be typed to AG unlike some previous studies such as Anguiz and Martin (1989), Bains and Bisht (1995) and Bandy and Leach (1988). Previous studies (Schneider *et al.,* 1997a; Carling and Leiner, 1986) have stated the difficulty of identifying AG2-1 through observation of hyphal fusion, as hyphal fusion categories C1, C2 and C3 were all present between AG2-1, in this study hyphal fusion reaction C1 and C2 were present in fusion, suggesting these isolates are less related genetically but also as C1 reaction can occur between AGs meaning that AG typing is uncertain. AG2-1 identification in this study was confirmed using primers for AG2-1 designed by Carling *et al.* (2002a).

As well as in hyphal fusion reaction type, diversity in AG2-1 was evident in the hyphal growth rates, pathogenicity tests and length of the rDNA IGS1 region. Amongst isolates of AG2-1, isolates could have one of three lengths of IGS1 region, isolates of AG2-1 were designated to 's', 'n' and 'l' groups based on the length of the IGS1 region. Glasshouse and field experiment data showed that differences between the IGS1 groups of AG2-1 to cause stem canker. The AG2-1 's' isolates caused slight infection only capable of causing narrow lesions no longer than 5 mm, whilst isolates of AG2-1 'n' caused severe stem canker infections, similar to that of AG3 and AG5. Infections initiated by AG2-1 'l' were intermediate of the two types.

Analysis of the ITS region showed that the AG2-1 's' isolates clustered together, indicating they may be a definitive subgroup of AG2-1. Comparison of ITS sequences showed that isolates of AG2-1 'n' and AG2-1 'l' were not clustered into separate groups. The isolate, X1, of AG2-1 'l' tested in glasshouse experiments, may be an avirulent member of the group. There is also only a slight difference between 'n' and 'l' IGS1 lengths (approximately 20 bp) as opposed to between 'n' and 's'. As few isolates of the 'l' type were available in this study the 20 bp difference may be variation in a group separate to the AG2-1 isolates possessing the shorter IGS1 region.

Further investigation is warranted here to study the diversity of AG2-1 isolates and the implications of such diversity. Sequencing more genes from AG2-1 isolates and determining the pathogenicity of these isolates to other hosts. perhaps the isolates of AG2-1 's' are highly pathogenic to other hosts and

have mechanism of virulence adapted to that host. AG2-1 is known to infect a variety of hosts including brassicas, lettuce, cereals, tulips, spinach and sugar beet (Tu *et al.,* 1996) and this may have implications in rotation strategies.

The speculation that fungicides, which are selective for specific AGs, could increase the frequency of AGs capable of causing greater disease severity was examined in this study, and also if resistance has developed to fungicides widely used to control rhizoctonia potato disease. An assay to determine the EC_{50} of *R. solani* isolates to several fungicides indicated that no AGs associated with potato were resistant to fungicides used for rhizoctonia potato disease control. However, isolates of AG5 and AG8 shown to be insensitive to the fungicide pencycuron, which is concordant with previous studies (as reviewed by Kataria and Gisi, 1999).

Pencycuron is commonly used as a seed treatment in the UK. In this investigation only one isolate of AG5 and no isolates of AG8 were found in potato crops. Pencycuron has been used as a seed treatment since the late 1980s, if it was effecting the proportions of AGs present in potatoes then it could be expected that AG5 and AG8 would be better represented in this study. Therefore, it can be concluded that the use of pencycuron to date (2004) has not caused a change in the potato population in *R. solani*, this may be because pencycuron is widely used as a seed treatment and, as indicated in the field experiments in this study, AG5 and AG8 are not prolific producers of sclerotia on potato.

Determination of the EC_{50} for fungicides also revealed that isolates of AG1-IC and AG2-3 were insensitive to pencycuron, which has not been observed before. In this study and previous studies (Kataria and Gisi, 1999) isolates of AG4 were either sensitive or insensitive to pencycuron. Kim and Yamaguchi (1996) suggested that this may correspond with the subgroups of AG4. Subsequently, isolates used by Kim and Yamaguchi (1996) have been assigned to subgroups (Boysen *et al.*, 1996), matching the isolates used in these studies have indicated that AG4 HG-I is sensitive to pencycuron whilst AG4 HG-II is insensitive to pencycuron. Also, analysis of rDNA ITS sequences in this study, has also revealed that potato isolates of AG4 belong to the subgroup HG-I, this suggests that pencycuron can also be used to control rhizoctonia potato disease in areas where AG4 is present.

The use of rDNA ITS sequences are useful in the study of *R. solani* and can be used to determine the subgroup of an isolate. In this case the ability to correlate subgroup with fungicide sensitivity provides information on the effectiveness of the use of pencycuron to treat AG4 on potatoes. In this study, analysis of rDNA ITS sequences allowed the construction of phylogenetic trees indicating relationships between isolates of AG2-1, 3, 5 and 8 and confirming the existence of subgroups within those AGs.

The rDNA sequences obtained in this study and those present on GenBank were also used to design primers for use in a diagnostic assay to detect the presence of AG3PT in soil, plant or culture. However this assay, when used in conjunction with internal standards was not able to quantify the amount of *R.*

solani AG3PT present in soil. This is likely to be because very low levels of target DNA were extracted from the soil and were co-extracted with PCR-inhibitory compounds. Future attempts to quantify *R. solani* AGs in soil should utilise real-time technology, which is more sensitive than competitive PCR (Schena *et al.,* 2004). The methods of DNA extraction used in this study may be suitable for use with such an assay, particularly the guanidinium thiocyanate method, which offers the advantage of possibly being suitable for high throughput use as the method does not require DNA pelleting, unlike the other methods. However addition of a baiting step offers the advantage of detecting infectious units rather than *R. solani* soil DNA. The presence of different fungal structures may cause an inconsistent relationship between DNA concentration and infection potential of *R. solani* in soil. The design of any diagnostic assay to quantify *R. solani* in soil must consider AG. Profound differences in pathology, epidemiology and biology exist between AGs, and therefore knowledge of which AG is present is of major importance when implementing disease control strategies for rhizoctonia disease or conducting research into any aspect of the fungus.

8.2 Further research

- Varietal resistance to rhizoctonia potato disease

Wastie (1994) stated that diversity of *R. solani* may have caused problems in resistance breeding programmes. Considering AG may help to explain the presence of inconsistencies in such work. In this study, no tuber deformations were observed; unlike in Campion *et al.* (2003) who, particularly with isolates

of AG2-1 and AG5, found that *R. solani* could cause tuber deformations. The cultivar Désirée was used for all experiments in this investigation, unlike in Campion *et al.* (2003) where cultivars Bintje, Cynthia, Marine, Monalisa and Samba were tested. Further work could test for varietal resistance to all AGs considering all phases of the disease: stem and root infections, black scurf and tuber deformations. Analyzing the mechanisms of virulence between AGs may also provide useful clues into novel methods of control, for example understanding what makes AG3PT produce more sclerotia on potato than other AGs may give useful insights into managing black scurf.

- Development of a diagnostic assay for multiple AGs of *R. solani*

Consideration of AGs must also be made in the design of a successful diagnostic system for rhizoctonia potato disease. An assay could be designed, which detects and quantifies all AGs present in a soil sample. This assay could also be designed to distinguish between subgroups, for example AG2-1 's' and AG2-1 'n' which differ in virulence. The assay should be based on real-time PCR technology. This assay, in conjunction with further experiments data comparing disease development between different AGs with different levels of inoculum, could determine the relative risk of disease. With knowledge of this risk, growers could decide whether or not to plant, or implement the appropriate control strategy.

- Significance of other hosts in rhizoctonia potato disease

This study has indicated that sugar beet may be associated with *R. solani* AG3PT and couch grass is associated with AG5. Further work could

determine the ability of other plants to transmit the disease or increase the amount of *R. solani* potato disease present in the soil. Such studies could involve survey work, sampling weed and other crop species, isolating any *R. solani* present and determine the AG. Alternatively, diagnostic assays could be used to screen these plants to determine the presence of AGs associated with potato. Crop rotation field experiments could also be undertaken, which consider several AGs, and would determine the optimum rotation sequence to manage infections of soil associated with a particular AG. It would also be interesting to screen many plant species to determine susceptibilities to AG2-1 subgroups, AG5 and AG8. This may indicate if these AGs have a primary host which could be avoided. It may also indicate if the subgroups of AG2-1 have evolved specificity to different hosts.

- Significance of root infection on potato yield

This study has revealed that root infection can play a major role in determining yield losses. The study of root infections is perhaps under studied due to the difficulty in observing the interaction beneath the soil. Further work could investigate root infection in detail using glasshouse and field experiments to study root infections caused by AG3PT, AG8 and perhaps other potato pathogens. Soil sampling and a diagnostic assay for AG8 may also determine the significance of AG8 root infections.

- Survey of incidence of individual AGs over several years

The use of a diagnostic assay to detect AGs could be used in a survey over several years from perhaps soil and potato samples. Over several years, with

several hundred samples, it may be possible to determine if conditions such as temperature or rainfall influence the proportion of AGs present. Soil sampling would also determine if other AGs other than the ones present in this study are also present in British soils.

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Appendix 1. Date of isolation, anastomosis group, IGS1 length for each isolate with the symptom, variety and county of origin of the potato sample

Isolate	Date of isolation	Anastomosis group	IGS1 length (bp)	Symptom	Variety	County of origin
X1	May 2001	2-1	570	Black scurf	Premiere	Shropshire
X2	June 2001	3	680	Stem lesion	Sante	Shropshire
X3	June 2001	3	680	Stem lesion	Premiere	Shropshire
X4	June 2001	3	680	Root lesion	Premiere	Shropshire
X5	June 2001	3	680	Stem lesion	Lady Rosetta	Shropshire
X6	June 2001	3	680	Black scurf	Lady Rosetta	Shropshire
X7	June 2001	3	680	Stem lesion	Lady Rosetta	Shropshire
X8	June 2001	3	680	Stem lesion	Saturna	Shropshire
X9	July 2001	3	680	Stem lesion	Nadine	Norfolk
X10	July 2001	3	680	Root sclerotia	Harmony	Norfolk
X11	July 2001	3	680	Stem lesion	Nadine	Norfolk
X12	July 2001	3	680	Stem lesion	Premiere	Norfolk
X13	July 2001	3	680	Root sclerotia	Premiere	Norfolk
X14	July 2001	3	680	Stem lesion	Pentland Dell	Norfolk
X15	July 2001	3	680	Root sclerotia	Pentland Dell	Norfolk
X16	July 2001	3	680	Stem lesion	Marfona	Cambridgeshire
X17	July 2001	3	680	Root sclerotia	Désireé	Shropshire
X18	July 2001	3	680	Stem lesion	Désireé	Shropshire
X19	July 2001	3	680	Stolon lesion	Marfona	Shropshire
X20	July 2001	3	680	Root sclerotia	Maris Piper	Norfolk
X21	July 2001	3	680	Stem lesion	Asterix	Norfolk
X22	July 2001	3	680	Stolon lesion	Marfona	Suffolk
X23	July 2001	3	680	Stem lesion	Marfona	Suffolk
X24	July 2001	3	680	Stem lesion	Estima	Scotland (Tayside)
X25	July 2001	3	680	Root sclerotia	Estima	Scotland (Tayside)
X26	July 2001	3	680	Black scurf	Estima	Shropshire
X27	July 2001	3	680	Black scurf	Sante	Shropshire
X28	July 2001	3	680	Black scurf	Sante	Shropshire
X29	July 2001	3	680	Stem lesion	Lady Rosetta	Cornwall
X30	July 2001	3	680	Black scurf	Combo	Lancashire
X31	July 2001	3	680	Stem lesion	Nadine	Unknown
X32	July 2001	3	680	Stem lesion	Cultra	Scotland (Tayside)
X33	July 2001	3	680	Stem lesion	Amour	Scotland (Tayside)
X34	July 2001	3	680	Stem lesion	Estima	Scotland (Tayside)
X35	July 2001	3	680	Stem lesion	Combo	Lancashire
X36	July 2001	3	680	Root lesion	Combo	Lancashire
X37	July 2001	3	680	Stem lesion	Maris Piper	Lancashire
X38	July 2001	3	680	Root lesion	Maris Piper	Lancashire
X39	July 2001	3	680	Stem lesion	Marfona	Shropshire
X40	Aug 2001	3	680	Stem lesion	Nadine	Lancashire
X41	Aug 2001	3	680	Stem lesion	Nadine	Lancashire
X42	Aug 2001	3	680	Stem lesion	Maris Bard	Lancashire
X43	Aug 2001	3	680	Root sclerotia	Maris Bard	Lancashire
X44	Aug 2001	3	680	Black scurf	Maris Bard	West Yorkshire
X45	Aug 2001	3	550	Stem lesion	Maris Bard	West Yorkshire
X46	Aug 2001	2-1	680	Stolon lesion	Unknown	Cheshire
X47	Aug 2001	3	680	Stem lesion	Nadine	Northumberland
X48	Aug 2001	3		Stem sclerotia	Nadine	Northumberland

Isolate	Date of isolation	Anastomosis group	IGS1 length (bp)	Symptom	Variety	County of origin
X49	Aug 2001	3	680	Black scurf	Nadine	Northumberland
X50	Sep 2001	3	680	Black scurf	Premiere	Scotland
X51	Sep 2001	3	680	Black scurf	Charlotte	Unknown
X52	Sep 2001	2-1	550	Stem lesion	Maris Bard	Scotland (Angus)
X53	Sep 2001	3	680	Black scurf	Désireé	Scotland
X54	Sep 2001	3	680	Black scurf	Juliette	Suffolk
X55	Sep 2001	3	680	Black scurf	Juliette	Suffolk
X56	Sep 2001	3	680	Black scurf	Charlotte	Cornwall
X57	Sep 2001	3	680	Black scurf	Juliette	Suffolk
X58	Sep 2001	3	680	Black scurf	Violette	Suffolk
X59	Sep 2001	3	680	Black scurf	Comet	Suffolk
X60	Oct 2001	3	680	Black scurf	Nadine	Lancashire
X61	Oct 2001	3	680	Black scurf	Charlotte	Lincolnshire
X62	Oct 2001	3	680	Black scurf	Nicola	Suffolk
X63	Oct 2001	3	680	Black scurf	Charlotte	Northumberland
X64	Nov 2001	3	680	Black scurf	Désireé	Shropshire
X65	Nov 2001	3	680	Black scurf	Romano	Pembrokshire
X66	Nov 2001	3	680	Black scurf	Charlotte	Somerset
X67	Nov 2001	3	680	Black scurf	Romano	Scotland
X68	Nov 2001	3	680	Black scurf	Cara	Somerset
X69	Nov 2001	3	680	Black scurf	Romano	Devon
X70	Nov 2001	3	680	Black scurf	Estima	Herefordshire
X71	Nov 2001	3	680	Black scurf	Romano	Devon
X72	Nov 2001	3	680	Black scurf	Nicola	Scotland
X73	Nov 2001	3	680	Black scurf	Sante	Worcestershire
X74	Nov 2001	3	680	Black scurf	Cara	Somerset
X75	Nov 2001	3	680	Black scurf	Sante	Oxfordshire
X76	Nov 2001	3	680	Black scurf	Nicola	North Yorkshire
X77	Nov 2001	3	680	Black scurf	Maris Peer	Shropshire
X78	Nov 2001	3	680	Black scurf	Désireé	Staffordshire
X79	Dec 2001	3	680	Black scurf	Désireé	Essex
X80	Dec 2001	3	680	Black scurf	Désireé	Shropshire
X81	Dec 2001	2-1	510	Black scurf	Désireé	Scotland
Y1	May 2002	3	680	Stem lesion	Rocket	Shropshire
Y2	May 2002	2-1	510	Black scurf	Rocket	Shropshire
Y3	June 2002	2-1	550	Stem lesion	Estima	Lincolnshire
Y4	June 2002	3	680	Black scurf	Maris Piper	Essex
Y5	June 2002	3	680	Black scurf	Estima	Shropshire
Y6	June 2002	3	680	Stem lesion	Asterix	Norfolk
Y7	June 2002	3	680	Stem lesion	Lady Rosetta	Northumberland
Y8	June 2002	3	680	Stem lesion	Lady Rosetta	Northumberland
Y9	June 2002	3	680	Stem lesion	Saturna	Norfolk
Y10*	June 2002	3	680	Stem lesion	Saturna	Shropshire
Y11	June 2002	3	680	Stem lesion	International Kidney	Shropshire
Y12	June 2002	3	680	Stem lesion	Pentland Dell	Cheshire
Y13	June 2002	3	680	Stem lesion	Pentland Dell	Cheshire
Y14	June 2002	3	680	Stem lesion	Pentland Dell	Cheshire
Y15	June 2002	3	680	Stem lesion	Maris Piper	Cheshire
Y16	June 2002	3	680	Stem lesion	Maris Piper	Cheshire
Y17	June 2002	3	680	Stem lesion	Maris Piper	Cheshire
Y18	June 2002	3	680	Stem lesion	Maris Piper	Cheshire
Y19	June 2002	3	680	Stem lesion	Maris Piper	Cheshire

Isolate	Date of isolation	Anastomosis group	IGS1 length (bp)	Symptom	Variety	County of origin
Y21	June 2002	3	680	Stem lesion	Maris Piper	Suffolk
Y22	June 2002	3	680	Stem lesion	Dundrod	Staffordshire
Y24	July 2002	3	680	Stolon lesion	Saturna	Norfolk
Y25	July 2002	2-1	550	Stem lesion	Saturna	North Yorkshire
Y29	July 2002	3	680	Stem lesion	Nadine	Shropshire
Y37	July 2002	3	680	Stem lesion	Saturna	Norfolk
Y41	July 2002	3	680	Black scurf	Maris Bard	Shropshire
Y42	Aug 2002	3	680	Black scurf	Winston	Cambridgeshire
Y43	Sept 2002	3	680	Black scurf	Estima	Shropshire
Y44	Oct 2002	3	680	Black scurf	Juliette	Norfolk
Y45	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y46	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y47	Oct 2002	3	680	Black scurf	Juliette	Norfolk
Y48	Oct 2002	3	680	Black scurf	Juliette	Norfolk
Y49	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y50	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y51	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y52	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y53	Oct 2002	3	680	Black scurf	Juliette	Essex
Y54	Oct 2002	3	680	Black scurf	Juliette	Suffolk
Y55	Oct 2002	5	520	Black scurf	Premiere	Norfolk
Y56	Oct 2002	3	680	Black scurf	Hermes	Norfolk
Y57	Nov 2002	3	680	Black scurf	Premiere	Shropshire
Y58	Dec 2002	3	680	Black scurf	Estima	Shropshire
Y59	Dec 2002	3	680	Black scurf	Spey	Shropshire
Y60	Dec 2002	3	680	Black scurf	International Kidney	Jersey
Y61	Dec 2002	3	680	Black scurf	International Kidney	Jersey
Y62	Dec 2002	3	680	Black scurf	Maris Piper	Shropshire
Y63	Dec 2002	2-1	510	Black scurf	Unknown	Scotland
Y64	Dec 2002	3	680	Black scurf	Maris Peer	Essex
Z1	Apr 2003	2-1	510	Black scurf	Estima	Scotland
Z2	Apr 2003	3	680	Black scurf	Maris.Piper	Shropshire
Z3	Apr 2003	3	680	Black scurf	Cosmos	Scotland
Z4	Apr 2003	3	680	Black scurf	Nicola	Scotland
Z5	Apr 2003	3	680	Black scurf	Kind Edward	Lancashire

*This isolate was found in the same sample as the couch grass isolate T1.

Appendix 2. Husbandry practices adopted in the 2001 field trial

Situated at Swans Leasow field, Harper Adams University College
Ordnance survey map grid reference: SJ71851995

Date	Operation
11/04/2001	Sub-soiling
12/04/2001	Plough
28/04/2001	Hydro granular (nitrogen 12%, phosphorus 12%, potassium 25%) 1000 kg ha^{-1}
28/04/2001	Big ridges pulled up
24/05/2001	Bed tilled
24/05/2001	Destoned
25/05/2001	Hand planting of seed tubers (Désirée, SE2 grade 35-55 mm)
05/06/2001	Gramoxone 100 (Paraquat 200 g l^{-1}) 2 l/ha and bullet (150 g l^{-1} cyanazine and 264 g l^{-1} pendimethalin) 5 l ha^{-1}
20/06/2001	Hand weeding of plots
28/06/2001	Nitram (34.5 % Nitrogen) 370 kg ha^{-1}
02/07/2001	Trustan (3.2% cymoxanil, 56% mancozeb and 8% oxadixyl w/w) 2.5 kg ha^{-1}
06/07/2001	Titus (rimsulfuron 25% w/w) 40 g ha^{-1} + nion (90% ethylene oxide condensate) 10 ml l^{-1}
09/07/2001	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2 kg ha^{-1}
12/07/2001	Samples taken for disease assessment (hand lifting)
16/07/2001	Trustan (3.2% cymoxanil, 56% mancozeb and 8% oxadixyl w/w) 2.5 kg ha^{-1}
24/07/2001	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2 kg ha^{-1}
24/07/2001	Aphox (50% primicarb w/w) 280 g ha^{-1}
01/08/2001	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2 kg ha^{-1}
01/08/2001	Aphox (50% primicarb w/w) 280 g ha^{-1}
14/08/2001	Opie WP (mancozeb 80%) 1.7 kg ha^{-1}
23/08/2001	Shirlan programme (fluazinam 500 g l^{-1}) 300 ml ha^{-1}
23/08/2001	Aphox (50% primicarb w/w) 280 g ha^{-1}
01/09/2001	Shirlan programme (fluazinam 500 g $^{-1}$l) 300 ml ha^{-1}
01/09/2001	Aphox (50% primicarb w/w) 280 g ha^{-1}
14/09/2001	Supertin 4L (fentin hydoxide 480 g/l) 0.56 l ha^{-1}
14/09/2001	Reglone (diquat 200g/l) 4 l ha $^{-1}$and flail topping
03/10/2001	Hand lifted

Appendix 3. Husbandry practices adopted during the 2003 field trial

Situated at First Foxhole field, Harper Adams University College
Ordnance Survey map grid reference: SJ71401995

Date	Operation
17/03/2003	Sub-soiling
07/04/2003	Plough
24/04/2003	Omex (14% nitrogen, 5% phosphorus 20%, potassium) 1785 kg ha^{-1}
27/04/2003	Big ridges
06/05/2003	Bed tilled
06/05/2003	De-stoned
06/05/2003	Pulled up ridges
09/05/2003	Hand planting of seed tubers (Désirée, VTSC2 grade 35-55 mm)
30/05/2003	PDQ (4.5 g l^{-1} diquat and 120 g l^{-1} paraquat) 2.5 l ha^{-1} and Alpha cinuron 50SC (linuron 500 g l^{-1}) 2.5L ha^{-1}
16/06/2003	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2kg ha^{-1} / Trustan 2.5kg ha^{-1}
24/06/2003	Invader (7.5% dimethomorph and 66.7% mancozeb w/w) 2kg ha^{-1}
24/06/2003	Calcium nitrate tropicote (15.5% nitrogen and 26% CaO) 400kg ha^{-1}
01/07/2003	Samples taken for disease assessment (hand lifting)
07/07/2003	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2kg ha^{-1}
23/07/2003	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2kg ha^{-1}
08/08/2003	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2kg ha^{-1}
20/08/2003	Curzate M68 (4.5% cymoxanil and 68% mancozeb w/w) 2kg ha^{-1}
02/09/2003	Shirlan (fluazinam 500 g/l) 0.3L ha^{-1}
02/09/2003	Reglone (diquat 200g l^{-1}) 4 L ha^{-1} and flail topping
25/09/2003	Hand lifted